Analysis of the *in vivo* functions and assembly pathway of small subunit ribosomal proteins in *Saccharomyces cerevisiae*

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.) DER
NATURWISSENSCHAFTLICHEN FAKULTÄT III – BIOLOGIE UND
VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von
Sébastien Ferreira-Cerca
aus Rochefort s/Mer, Frankreich

Regensburg, November 2007

Promotionsgesuch eingereicht am: 14 November 2007

Die Arbeit wurde angeleitet von: Prof. Dr. Herbert Tschochner und Prof. Dr. Pierre-Emmanuel Gleizes.

Prüfungausschuss:

Vorsitzender:	Prof. Dr. Gernot Längst
1. Gutachter:	Prof. Dr. Herbert Tschochner
2. Gutachter:	Prof. Dr. Pierre-Emmanuel Gleizes
3. Prüfer:	Prof. Dr. Thomas Dresselhaus

Tag der mündlichen Prüfung: 15 Februar 2008

Diese Arbeit wurde von Oktober 2003 bis November 2007 unter der Anleitung von Dr. Philipp Milkereit, Prof. Dr. Herbert Tschochner (Universität Regensburg, Deutschland) und Prof. Dr. Pierre-Emmanuel Gleizes (Université Toulouse, Frankreich) am Lehrstuhl für Biochemie III des Instituts für Biochemie, Genetik und Mikrobiologie der Universität Regensburg angefertigt.

Ich erkläre hiermit, dass ich diese Dissertation selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet haben. Diese Arbeit war bisher noch nicht Bestandteil eines Prüfungsverfahrens, andere Promotionsversuche wurden nicht unternommen.

Regensburg, 25 Februar 2008

À la mémoire de mon père,

TABLE OF CONTENTS

SUMMARY

In eukaryotes, *in vivo* formation of the two ribosomal subunits from four ribosomal RNAs (rRNAs) and approximately 80 ribosomal proteins (r-proteins) involves more than 150 non-ribosomal proteins and around 100 small non-coding RNAs. Ribosome biogenesis is temporally and spatially organised within three different cellular compartments: the nucleolus, nucleoplasm and cytoplasm.
Despite the rising knowledge about ribosome function and structure and how ribosomal subunits assemble *in vitro* in bacteria, the *in vivo* role of many ribosomal proteins remains obscure both in pro- and eukaryotes.

This work describes the systematic analysis of yeast small subunit r-proteins (rpS) *in vivo* function(s) in small ribosomal subunit (SSU) maturation and assembly.

The results described herein demonstrate that most eukaryotic r-proteins fulfill different roles in ribosome biogenesis, making them indispensable for growth. Different r-proteins control distinct steps of nuclear and cytoplasmic pre-18S rRNA processing and, thus, ensure that only properly assembled ribosomes become engaged in translation. Furthermore several r-proteins are required for efficient nuclear export of pre-18S rRNA, suggesting that they form an interaction platform with the export machinery.
In addition, *in vivo* analysis of rpS assembly suggests that the pre-rRNA – rpS interactions are stabilised in the course of the SSU maturation process.
Finally, analysis of rpS assembly status in two mutants in which pre-SSU nuclear export is blocked (*crm1*, *rps5*) and one in which export is strongly delayed (*rps15*) show that key aspects of the *in vivo* assembly of eukaryotic r-proteins into distinct structural parts of the SSU are similar to the *in vitro* assembly pathway of their prokaryotic counterparts. Interestingly, the establishment of a stable assembly intermediate of the eukaryotic SSU-body, but not of the SSU-head, is closely linked to early rRNA processing events. On the other hand, formation of assembly intermediates of the head controls efficient nuclear export of the SSU and cytoplasmic pre-rRNA maturation steps.
Therefore the formation of certain assembly intermediates is required to allow the rRNA processing steps or the export of the SSU to take place. The above intermediates can also contribute substantially to the quality control of the maturing subunit.

RÉSUMÉ

Chez les cellules eucaryotes, la formation des deux sous-unités ribosomiques comportant au total quatre ARN ribosomiques (ARNr) et environ 80 protéines ribosomiques nécessite plus de 150 facteurs non-ribosomiques et environ 100 petits ARN non codant. La biogenèse des ribosomes est temporellement et spatialement organisée dans trois compartiments cellulaires différents: le nucléole, le nucléoplasme et le cytoplasme.
Alors que de nombreuses données existent quant à la structure et la fonction des ribosomes, ainsi que l´assemblage des protéines ribosomiques *in vitro* chez les bactéries, le rôle *in vivo* de nombreuses protéines ribosomiques reste obscure chez les cellules pro- et eucaryotes.

Cette thèse décrit l´analyse systématique de(s) la fonction(s) des protéines ribosomiques dans la maturation et l´assemblage de la petite sous-unité du ribosome (SSU) *in vivo* chez la levure.

Les résultats décrits ci-après démontrent que la plupart des protéines ribosomiques, chez les eucaryotes, jouent différents rôles importants au cours de la biogenèse de la petite sous-unité du ribosome, les rendant indispensables pour la survie cellulaire. Différentes protéines ribosomiques contrôlent des étapes distinctes de la maturation nucléaire et cytoplasmique des précurseurs de l´ARNr de 18S, et de ce fait, assurent que seuls les ribosomes proprement assemblés sont engagés dans le processus de traduction. De plus, plusieurs protéines ribosomiques sont requises pour permettre l´export efficace des particules de pré-40S, suggérant que ces protéines forment une plateforme d´interaction avec la machinerie d´exportation.
Par ailleurs, l´analyse *in vivo* de l´assemblage des protéines ribosomiques de la petite sous–unité suggère que les intéractions entre les pré-ARNr et les protéines ribosomiques sont stabilisées au cours du processus de maturation de la petite sous unité du ribosome.
En outre, l´analyse *in vivo* du statut de l´assemblage des protéines ribosomiques de la petite sous-unité, dans deux mutants chez lesquels l´export nucléaire des particules de pré-40S est bloqué (*crm1, rps5*) et dans un mutant (*rps15*) chez lequel cet étape est fortement retardée, démontre que les étapes clé de l´assemblage des protéines ribosomiques chez les eucaryotes dans des structures distinctes de la sous-unité 40S (corps et tête) sont similaires aux étapes d´assemblage mises en évidence *in vitro* pour les protéines ribosomiques homologues chez les procaryotes. Finalement, chez les eucaryotes l´établissement d´un intermédiaire structural

stable du corps de la petite sous-unité, mais pas de la tête, est étroitement lié aux étapes précoces de maturation du pré-ARNr. De plus, la formation d´un intermédiaire de la tête contrôle l´efficacité de l´export nucléaire de la petite sous-unité du ribosome et les étapes cytoplasmique de maturation du pré-ARNr.

En conséquence, la formation de certain intermédiaire structuraux est requise pour permettre aux étapes de maturation de l´ARNr et d´export de la petite sous-unité du ribosome d´avoir lieu. De plus, ces intermédiaires peuvent contribuer au contrôle qualité de la sous-unité du ribosome en cours de maturation.

ZUSAMMENFASSUNG

Die beiden Untereinheiten der eukaryotischen Ribosomen setzen sich aus vier ribosomalen RNAs (rRNAs) und ungefähr 80 ribosomalen Proteinen (r-Proteinen) zusammen. An ihrer Synthese, welche in drei verschiedenen zellulären Kompartimenten, dem Nukleolus, dem Nukleoplasma und dem Zytoplasma, stattfindet, sind mehr als 150 nicht-ribosomale Proteine und ca. 100 kleine, nicht-kodierende RNAs beteiligt.
Die Funktion der Ribosomen und ihre Struktur wurden bereits umfassend charakterisiert. Zudem gelang es, die Assemblierung prokaryotischer Ribosomen *in vitro* nachzuvollziehen. Hingegen war über die Funktionen der einzelnen ribosomalen Proteine in der Zelle bislang nur wenig bekannt.

In der vorliegenden Arbeit wurde daher mit Hilfe des eukaryotischen Modellorganismus *S.□erevisiae* systematisch analysiert, welche Funktionen den ribosomalen Proteinen der kleinen Untereinheit (rpS) *in vivo* bei der Reifung und Assemblierung der kleinen ribosomalen Untereinheit (SSU) zukommen.

Hierbei zeigte sich, dass die meisten eukaryotischen r-Proteine in der Ribosomenbiogenese für das Wachstum essenzielle Funktionen ausüben. Einige r-Proteine kontrollieren bestimmte Schritte der nukleären und zytoplasmatischen Prä-18S-rRNA-Prozessierung und garantieren auf diese Weise, dass nur korrekt zusammengebaute Ribosomen die Translation katalysieren.

Andere r-Proteine werden hingegen für den effizienten Export der Prä-18S-rRNA aus dem Kern benötigt und bilden dabei möglicherweise eine Interaktionsplattform mit der Export-Maschinerie aus.

Darüber hinaus lässt die *in vivo*-Analyse des SSU-Zusammenbaus vermuten, dass die Wechselwirkungen zwischen der Prä-rRNA uns den ribosomalen Proteinen im Laufe des SSU-Reifungsprozesses stabilisiert werden.

Schließlich erbrachte die Analyse von Mutanten, in denen der nukleäre Prä-SSU-Export entweder vollständig (*crm1*, *rps5*) oder weitestgehend (*rps15*) blockiert werden kann, dass die *in-vivo*-Assemblierung der r-Proteine in die kleine ribosomale Untereinheit in ähnlicher Reihenfolge abläuft wie für die Assemblierung *in vitro* der entsprechenden prokaryotischen r–Proteine zuvor ermittelt worden war. Interessanterweise wirkt sich die Ausbildung einer stabilen Assemblierungszwischenstufe des eukaryotischen SSU-„body“, nicht aber des SSU–„head“, auf frühe rRNA-Prozessierungsereignisse aus, wohingegen die Bildung von Assemblierungszwischenstufen des „head“ den SSU-Export aus dem Kern und zytoplasmatische rRNA-Reifungsschritte kontrolliert. Demzufolge ist die Ausbildung bestimmter Substrukturen oder Faltungsintermediate wichtig, damit definierte rRNA Prozessierungsschritte oder der Export der kleinen ribosomalen Untereinheit stattfinden kann. Sie tragen damit auch wesentlich zur Qualitätskontrolle der reifenden Untereinheit bei.

INTRODUCTION

1. The ribosome: structure and function.

Ribosomes are **r**ibo**n**ucleo-**p**rotein (**RNP**) complexes formed of several ribonucleic acid molecules (ribosomal RNA or **rRNA**) "decorated" with many proteins (ribosomal proteins or **r-proteins**).

In all living cells ribosomes constitute the heart of the translation machinery allowing conversion of the information encoded within the messenger RNA (mRNA), into amino acid chains: the proteins.

Thus, the ribosome is a fundamental macromolecular machinery participating in the flow of genetic information expression.

1.1. A brief history of protein synthesis research.

Ribosomes were first observed under the microscope by G. Garnier at the end of the 19th century, and described as a basophilic cytoplasmic component and termed ergastoplasm (for review see Haguenau, 1958). During the first half of the 20th century J. Brachet and others showed that the basophilic nature of the ergastoplasm is due to its RNA content (see Brachet, 1933 among others). Furthermore it became obvious that protein synthesis activity and the amount of cellular RNA were correlated (see Brachet, 1942 among others). In the mean time, particles containing most of the cellular RNA were isolated by high-speed centrifugation in different laboratories. A. Claude examined them by dark-field microscopy analysis (Claude, 1943), later on he termed these particles: "microsomes" (very likely to be ribosomes associated to endoplasmic reticulum, Claude, 1943). However, at this time no clear function could be assigned to these particles.

In the 1950s, *in vitro* incorporation of radioactive amino acids into nascent peptide chains using purified "microsomes" confirmed the anticipated role of these RNA containing particles in protein synthesis (Siekevitz and Zamecnick, 1951; Siekevitz, 1952). At the end of the 1950s, purified "microsomes" devoid of membrane fragments were termed "ribosomes" (Roberts, 1958) assuming the functional importance of the RNA contained in these particles. At the same time, F. Crick postulates the "central dogma" of biology concerning the flow of genetic information summarised in his assay on protein synthesis (Crick, 1958).

He and others suggested that «*It seems highly likely that the presence of RNA is essential for cytoplasmic protein synthesis*», «*there must be an RNA template in the cytoplasm*» and that «*template RNA' is located inside the microsomal particles* » (quoted from Crick, 1958). This generally accepted assumption suggested a model were ribosomal RNA would serve as template and led to the "one gene - one ribosome - one protein" hypothesis, neglecting in part the possible role of quickly metabolising RNA in protein synthesis.

At the beginning of the 1960s was established the role of "messenger RNA" to be a short living RNA playing the role of a transmitter of molecular information for protein synthesis (Crick et al., 1961; Jacob and Monod, 1961 among others).
Brenner et al performed metabolic labelling of bacteria with different radioisotopes before and after infection with bacteriophage T2. Interestingly, the neo-synthesis of bacteriophage specific proteins correlated with the association of newly synthesised RNA molecules with cellular ribosomes, while no *de novo* ribosome synthesis was observed. Suggesting the existence of a non-rRNA RNA molecule serving as template for protein synthesis (see Brenner et al., 1961).

In addition F. Crick suggested in the assay mention above that an adapter would be required to direct the assembly of amino acids into the correct order. This adapter model was further confirmed with the elucidation of the role of transfer RNA (tRNA) (Hecht et al., 1958a; Hecht et al., 1958b; Hoagland et al., 1958 among others). It was shown that in the course of protein synthesis, radio-labelled amino acids are transiently bound to a soluble low molecular weight RNA fraction: the tRNA (Hoagland et al., 1958).

At this stage of protein synthesis research, one remaining major black box was the "genetic code" governing the flow of genetic information via RNA to protein. The development of a reliable *in vitro* bacterial cell free translation system (Lamborg and Zamecnik, 1960) and the use of artificial RNA homo- and heteropolymers allowed to "*crack*" the genetic code (see Lengyel et al., 1961; Nirenberg and Matthaei, 1961 among others).

In summary, the ribosome is a RNP complex allowing the conversion of the genetic information encoded within mRNA, via codon-anticodon interaction with cognate aminoacyl-tRNA, into nascent polypeptide chains.

1.2. The ribosome: structure and composition.

1.2.1. Ribosome composition.

Two main types of ribosomes can be found in nature.

All prokaryotic organisms contain ribosomes with a sedimantation rate of **70S** (about 2.6 MDa with a mean diameter of 200 to 250 Å) which can be reversibly dissociated into a **50S LSU** (**l**arge **s**ub**u**nit) and a **30S SSU** (**s**mall **s**ub**u**nit) (Tissieres and Watson, 1958; Tissieres et al., 1959).
The **50S** ribosomal subunit is a RNP complex formed of two rRNAs - **23S** and **5S rRNA** - and about **32 r-proteins** in eubacteria, and slightly more in archaeabacteria. The **30S** ribosomal subunit is a RNP complex composed of one rRNA – the **16S rRNA** - and about **21 r-proteins** in eubacteria, and slightly more in archaeabacteria.

All eukaryotic organisms contain cytosolic ribosomes with a sedimantation rate of **80S** (about 4 MDa with a mean diameter of 250 to 300 Å) which can be reversibly dissociated into a **60S LSU** and a **40S SSU** (see Chao and Schachman, 1956; Chao, 1957; Chao, 1961 among others).
The **60S** ribosomal subunit is a RNP complex composed of three rRNAs - **25S** or **28S** in higher eukaryotes, a **5.8S** and **5S rRNA** - and around **46 r-proteins**. The **40S** ribosomal subunit is a RNP complex formed of one rRNA – **18S rRNA** - and around **32 r-proteins**.
In addition, eukaryotic cells contain also mitochondrial ribosomes however their size and composition is heterogeneous (Kitakawa and Isono, 1991; Nierhaus, 2004; Smits et al., 2007). In contrast, chloroplast ribosomes from higher plants belong to the group of 70S-like ribosomes (Nierhaus, 2004).

1.2.2. Structural features of ribosomes.

Despite slight differences - rRNA length, number of rRNAs and r-proteins - prokaryotic and eukaryotic ribosomes show striking morphological similarity with some variation in the different phylum (see Fig. 1A). Additionally, rRNAs secondary structures comparison are higly conserved between pro- and eukaryotes (see Fig. 1B and Gutell et al., 1985).

Furthermore, the high degree of conservation is also reflected on the r-protein level (see Lecompte et al., 2002 and Fig. 1C).

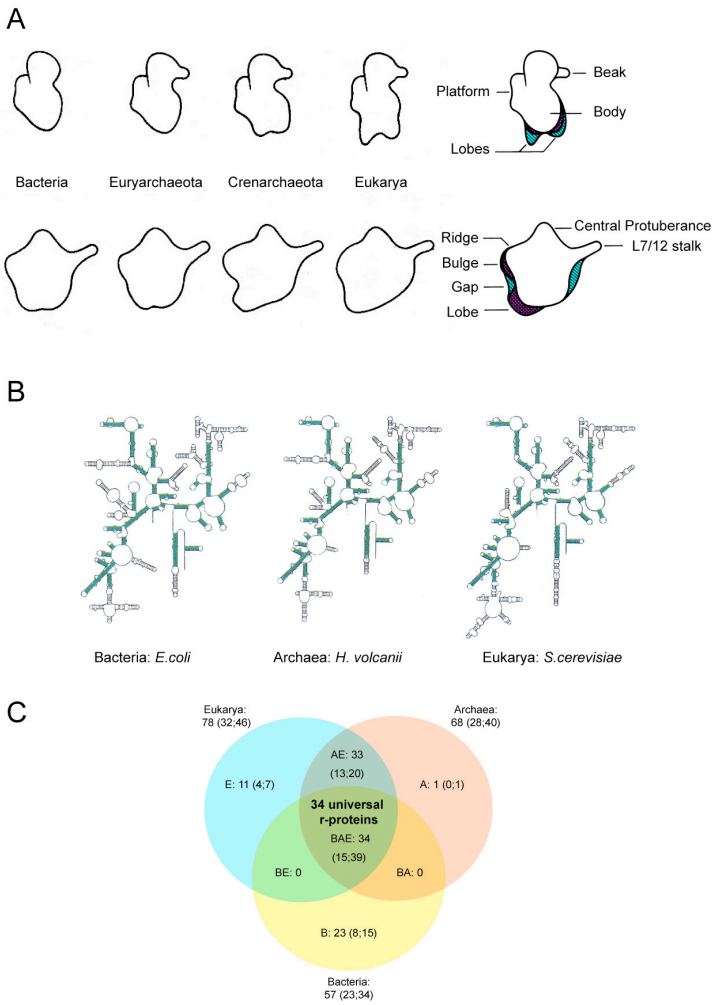


Figure 1: Conservation of the ribosomal subunits.

(A) Conserved ribosomal subunits morphology.

Diagram of low-resolution structures of ribosomal subunits from different organisms obtained from electron micrographs are shown. Reproduced and modified from Lake, 1985.

From left to right are shown the ribosomal subunits morphologies from *Synechocystis* 6701 (eubacteria); *H.□utirubrum* (euryarchaeota); *T. tenax* (crenarcheota); *S. cerevisiae* (eukarya). Upper and lower panel show the SSU and LSU morphologies respectively.

Right panel summarised the characteristic structural features found in the four ribosome types.

Features found in crenarcheota (but absent from both eubacteria and euryarchaeota) are indicated in purple. Eukaryotic features (abscent in prokaryotes) are indicated in blue.

(B) Conserved secondary structure of SSU rRNA. Adapted from Nierhaus, 2004.

The core structure (in green) common to all SSU rRNA from different representative organisms are shown.

(C) Conservation of r-proteins.

Venn´s diagram showing the general distribution of r-protein families between the three domains: Bacteria (B), Archaea (A), and Eukarya (E) adapted from Lecompte et al., 2002. The number of families is indicated for each set. The two numbers enclosed by parentheses refer to r-protein families found in the small and large ribosome subunits respectively.

Early RNA-RNA, RNA-protein cross-linking and protection experiments, neutron scattering, immuno-localisation, and electron microscopy analyses provided a first topology of the ribosome. More recently, structure of the eubacterial SSU and the prokaryotic LSU were revealed at the atomic level by X-ray crystallography (Cate et al., 1999; Agrawal et al., 2000; Ban et al., 2000; Svergun and Nierhaus, 2000; Wimberly et al., 2000; Noller et al., 2001; Yusupov et al., 2001; Brodersen et al., 2002; Wilson et al., 2002; Wilson and Nierhaus, 2003). However eukaryotic ribosome 3D structure lags behind and is so far based on cryo-EM analysis and homology modelling (Spahn et al., 2001). All the conserved r-proteins and rRNA segments could be positioned in this structure. However the archaeabacterial and eukaryote SSU specific counterparts and all the eukaryote specific LSU counterparts remain to be positioned in this structure (Spahn et al., 2001).

On the basis of morphological features visible in early electron micrographs, the SSU has been divided into an upper third, the **head**, and a lower two-third, the **body**. A deep cleft separates the head from a protrusion of the body called the **platform** (see Fig. 2 and 3B). Further structural landmarks were defined with the advent of cryo-EM techniques: the **beak** a protrusion of the head, the **neck**, and the **shoulder** at the interface between the head and the body and the **spur** at the bottom extremity of the body (see Brodersen et al., 2002).
Interestingly, phylogenic analysis of SSU structure shows extra electron densities, which are probably due to the presence of extra rRNA sequences and/ or extra r-proteins in archaeabacterial and eukaryotic ribosomes (Lake, 1985 and Fig. 1A). Interestingly, ribosomes isolated from crenoarchaeabacteria show an intermediate morphology between eubacterial and eukaryotic ribosomes (Lake, 1985 and Fig. 1A).

Eukaryotic SSUs show a strong subdivision at the bottom part of the body into a “left foot” and a “right foot”. Extra densities are also mainly present below the platform, forming the “left foot” and the “back lobe“ of the 40S subunit, at the “beak”, and at the solvent side of the head, building the “head lobe” (Spahn et al., 2001).

Based on the predicted secondary structure of 16S rRNA (Glotz and Brimacombe, 1980; Woese et al., 1980) the molecule can be divided into four main domains that radiate out from the central pseudoknot. These domains are termed the **5’, central, 3’major, and 3’ minor domains**. One remarkable feature of the 30S subunit structure is that the major morphological features (such as the head, platform, and body) are constructed largely by individual

secondary structure domains. The 5' domain makes up the bulk of the body, the central domain the platform, and the 3' major domain the entire head. The small 3' minor domain consists of just two rRNA helices located at the subunit interface.

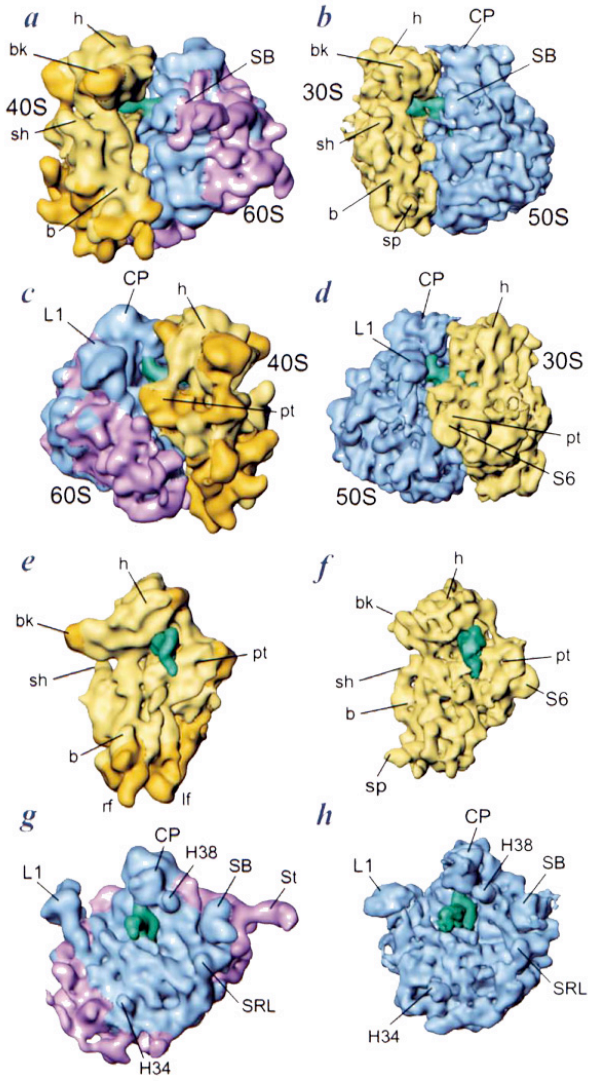


Figure 2: Comparison of the 80S ribosome from *S. cerevisiae* with the *E. coli* 70S ribosome. Reproduced from Spahn et al., 2001.

The cryo-EM map of the yeast 80S ribosome (a, c, e, and g) is shown together with the cryo-EM map of the *E.□oli* 70S ribosome (b, d, f, and h; Gabashvili et al., 2000). The ribosomes are shown from the L7/L12 side (a and b) and the L1 side (c and d). The computationally isolated small subunits (e and f) and large subunits (g and h) are shown from the interface side. The small subunits are in yellow, the large subunits in blue, and the P sitebound tRNA in green. Additional parts of the eukaryotic 80S ribosome that are due to expansion segments in the rRNAs and nonhomologous r-proteins are shown in gold (40S subunit) and purple (60S subunit). Landmarks for the 40S subunit: b, body; bk, beak; h, head; lf, left foot; rf, right foot; pt, platform; sh, shoulder; sp, spur. Landmarks for the 60S subunit: CP, central protuberance; L1, L1 protuberance; SB, stalk base; St, L7/ L12 stalk; H34, helix 34; H38, helix 38; SRL, sarcin-ricin loop.

The LSU is seen as a more compact structure, consisting of a **rounded base** with three almost cylindrical extensions. The three protuberances seen from the 50S side are called from the left to the right, the **L1 protuberance**, the **central protuberance (CP)**, and **L7/ L12 stalk** (according to *E. coli* r-protein nomenclature) (see Fig. 2). In addition, the **exit tunnel** is also seen at the solvent side of the LSU.

Interestingly, phylogenic analysis of LSU structure shows extra electron densities along the base of the subunit, which is probably due to the presence of extra rRNA sequences and/ or extra r-proteins in archaeabacterial and eukaryotic ribosomes (Lake, 1985 and Fig. 1A). Like for the SSU, LSU isolated from crenoarchaeabacteria show an intermediate morphology between eubacterial and eukaryotic ribosomes (Lake, 1985 and Fig. 1A).

In the 23S/ 25S rRNA secondary structure, the 5' and 3' terminal ends are brought together to form a helix (and circularise the rRNA). From this helix, 11 stem-loop structures are radiating, and organised into six different domains. Unlike the SSU, the rRNA domains of the LSU are difficult to assign to a specific structural landmark, since they have complicated, convoluted shapes, producing a compact, monolithic RNA mass (Ban et al., 2000; Nierhaus, 2004).

1.2.3. Ribosomal protein structures and their distribution in the ribosome.

Early neutron scattering, cross-linking, and immuno-locatisation experiments and the recent achievement of atomic resolution model of the ribosomal subunits, show that r-proteins from both subunits share common features.

Most of the r-proteins are located at the periphery on the surface of the ribosome structure, and are almost excluded from the subunit interfaces, and the peptidyl transferase centre. This global distribution in addition with mutant, and biochemical analyses lead to the conclusion that the ribosome is a ribozyme, since only the rRNA is directly involved in the catalysis of a peptide bond (Noller et al., 1992; Nitta et al., 1998; Hoang et al., 2004).

Ribosomal proteins of the large ribosomal subunit (**rpL**) are uniformely dispersed on the surface of the large subunit (Fig. 3A). In contrast, the vast majority of the ribosomal proteins of the small ribosomal subunit (**rpS**) are located on the back and surface of the small subunit, with a slightly higher density towards the top of the structure, due to the large number of r–proteins that bind to the SSU head (Fig. 3B).

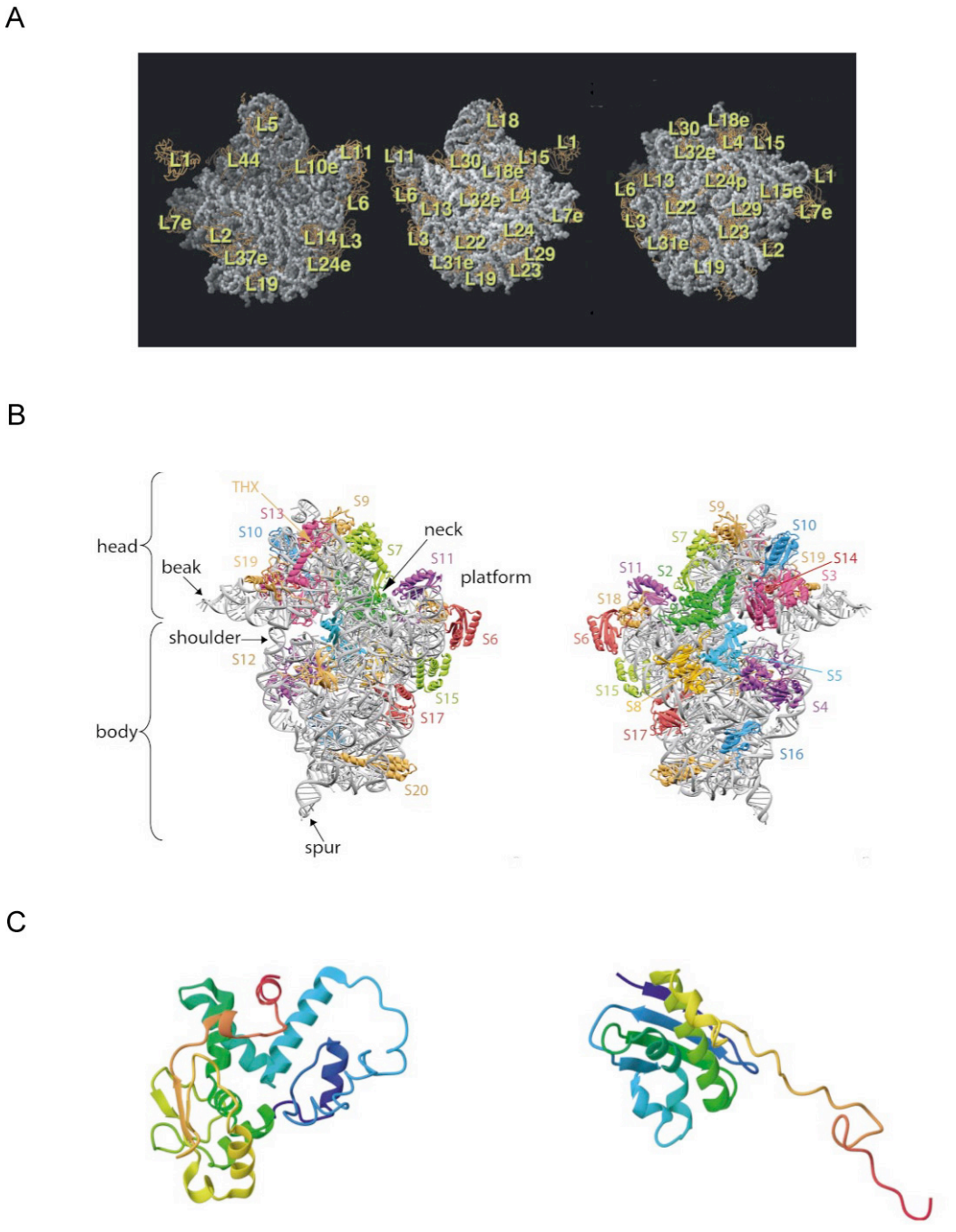


Figure 3: Spacial distribution of r-proteins on the ribosome surface.

(A) Localisation of the LSU r-proteins modified from Ban et al., 2000. The rRNA of the subunit is shown in gray and r-protein backbones are shown in gold. From left to right: the crown view of the subunit, the back side of the subunit in the 180° rotated crown view orientation, a view from the bottom of the subunit down the polypeptide tunnel exit which lies in the centre.

(B) Localisation of the SSU r-proteins reproduced from Brodersen et al., 2002. The 16S rRNA is shown in a gray ribbon-stick representation onto which each of the r-proteins is shown as a cartoon in color. Morphological features named in early EM reconstructions are indicated. From left to right: Front (or interface), side view of the subunit, and back side view respectively.

(C) Cartoon representation of r-proteins representative folding. Left part: globular folding, right part: globular fold and tail. Adapted from Brodersen et al., 2002.

The recent X-ray data were providing important informations concerning the 3D structures of prokaryotic r-proteins (more than 30 new atomic resolution structures).
The r-proteins typically have one or more globular domains. However, they also often contain extended internal loops or long N or C-terminal extensions that were often not seen in the isolated protein structures. These structures are intimately associated with the rRNA inside the ribosome (see for example Fig. 3C). These long extensions and loops are unusually rich in the basic side-chains arginine and lysine. Their role in rRNA-binding was originally predicted on the basis of asymmetric amino acid residues distribution observed along the primary sequences (Liljas, 1991).
In general, the globular domains are found mainly at the subunit surface, while the long extensions and loops are buried within the rRNA. Interestingly, the r-proteins are often bound to junctions between RNA helices, thereby often connecting separate domains (especially in the LSU).

In addition, many r-proteins have folding similarity and accordingly can be grouped into structural related families (see Brodersen et al., 2002; Klein et al., 2004).

1.3. Ribosome function.

The decoding of an mRNA transcript into protein by the ribosome can be divided into **three functional phases**: (1) recognition of the mRNA to be translated (**translation initiation**); (2) synthesis of a polypeptide chain according to the mRNA sequence (**translation elongation**); (3) and finally **translation termination** and recycling of the translation machinery.

With the help of biochemical, genetic and structural analyses both in pro- and eukaryotes the translation mechanism and its regulation was intensively studied. These mechanisms are extremely complex (for review see Nierhaus, 2004).

Recently, a snapshot of a significant intermediate state in protein synthesis was obtained by determining the structure of the 70S ribosome bound to three tRNAs. The three tRNAs are bound to **A**-, **P**- and **E**-sites (for **a**minoacyl-, **p**eptidyl- and **e**xit-sites respectively) that are located at the interfaces of both subunits. Each of the tRNA molecules bridges between the 30S and 50S ribosomal subunits. In the 30S, two of the three tRNAs are bound to the mRNA

fragment through codon-anticodon base pairing (in the A- and P-site). In the 50S subunit, the acceptor stem of the tRNA molecules occupying the A- and P-site converge in the **PTC** (**p**eptidyl **t**ransferase **c**entre) where the peptide bond is formed. Furthermore, the P-site is connected to an exit tunnel through which the nascent polypeptide leaves the ribosome

Hereafter will be a very short description about the current view of the main principles of protein synthesis.

1.3.1. Translation initiation (see Fig. 4A and B).

First, conserved **i**nitiation **f**actors IF3 or eIF 1,1A, 3 (**IFs** and **eIFs** initiation factor for pro- and eukaryotes respectively) bind to the 40S SSU (SSU*IF3 or SSU*eIF 1,1A, 3 complex), preventing association with the LSU in the absence of mRNA. Second, a 43S pre-initiation complex is formed by the interaction of the SSU*IF3 or SSU*eIF 1,1A, 3 complexes with a ternary complex consisting of the IF2/eIF2*GTP*Met-$tRNA_i^{Met}$ ($Met\text{-}tRNA_i^{Met}$= amino-acyl methionine charged initiator tRNA). Third, the pre-initiation complex binds to the mRNA.

In prokaryotes, many of the mRNAs contain upstream of each translation initiation codon a purine rich sequence called the Shine-Dalgarno (SD) sequence which is complementary to the 3' end of the 16S rRNA (anti-SD sequence) (among others Shine and Dalgarno, 1974; Chang et al., 2006). These interactions favour the correct positioning of the start codon in the P-site. However, it should be noted that in Gram-positive bacteria and archaeabacteria mRNA lacking this SD sequence also called leaderless mRNA are predominant and follow a different pathway (see for review Moll et al., 2002).

In eukaryotes, the 5' mRNA ends are modified with a 7-methylguanylated (m^7GpppN) cap structure (where N is any nucleotide) which is recognised by the eIF4 complex which in turn is recognised by the 43S pre-initiation complex and form a so-called 48S complex. Finally, the 40S ribosomal subunit scans the mRNA in an ATP dependent reaction in search of a start codon. A fraction of mRNA is translated independent of the cap-structure through recognition of a structured internal sequence in the mRNA upstream of the start codon, also known as **i**nternal **r**ibosome **e**ntry **s**ite or **IRES**.

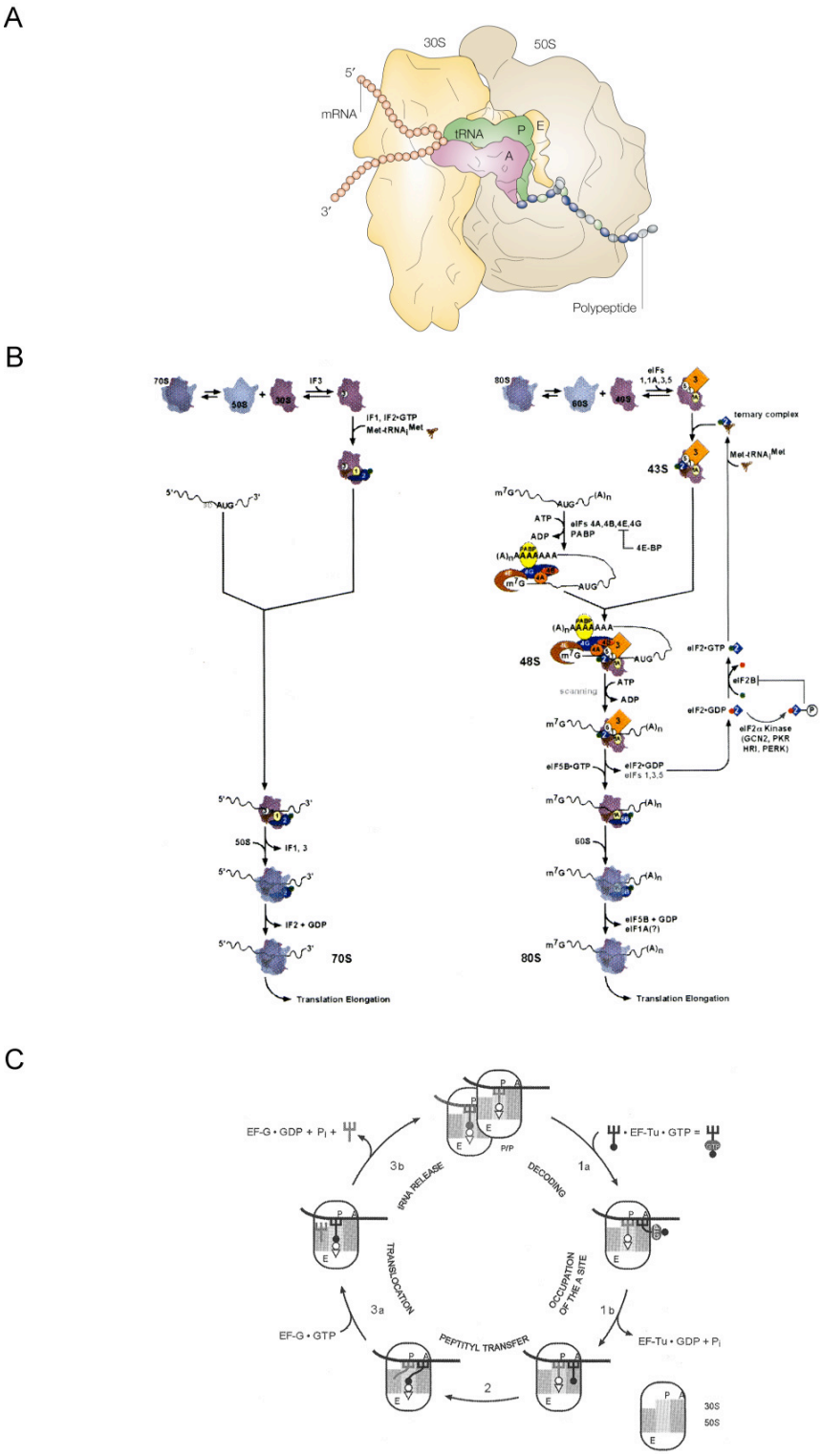


Figure 4: Ribosomes and protein synthesis.

(A) Translating ribosome. Reproduced from Lafontaine and Tollervey, 2001.

The three binding sites for tRNAs on the ribosome are shown. In the aminoacyl-site (A-site), a tRNA charged with an amino acid (aminoacyl-tRNA) is pairing with the messenger RNA (mRNA) being translated. The peptidyl-site (P-site) carries the growing peptide chain attached as a peptidyl-tRNA complex. The exit site (E-site) contains empty tRNAs on their way leaving the ribosome.

(B) Pathways of translation initiation in prokaryotes (left panel) and in eukaryotes (right panel) reproduced from Nierhaus, 2004.

The individual pathways have been aligned to reflect the conservation of the reactions and functions of the factors. The initiation factors are labelled IF and eIF for pro- and eukaryotes respectively.

Legend to Figure 4 (continued): At completion of the initiation pathway, Met-tRNA$_i^{Met}$ is bound to the ribosomal P-site and the A-site is vacant waiting for binding of the first elongating tRNA EF-Tu•GTP•aatRNA ternary complex.
(C) Model for the elongation cycle reproduced from Nierhaus, 2004. For details see introduction 1.3.2.

Finally, pairing of the anticodon of Met-tRNA$_i^{Met}$ with the AUG codon of the mRNA in the P-site triggers IF2/eIF2 GTP hydrolysis, and release almost all the IFs except IF2 or eIF5B (in pro- and eukaryotes respectively). This reaction promotes 60S ribosomal subunit joining to the pre-initiation complex. Subunit joining triggers GTP hydrolysis by IF2 or eIF5B (in pro- and eukaryotes respectively) and the subsequent release of these factors.

The 70S*mRNA or 80S*mRNA complexes are ready to enter the elongation phase of protein synthesis.

1.3.2. Translation elongation (see Fig. 4C).

Translation elongation requires the assistance of two highly conserved translation elongation factors EF-Tu, EF-G in bacteria and EF1A, EF2 in eukaryotes and archaeabacteria.
Translation elongation can be regarded as a cycle. First, an aa-tRNA*EF-Tu*GTP complex (aa for aminoacyl) is delivered to the empty A-site. If the cognate aa-tRNA is delivered, correct codon-anticodon pairing triggers GTP hydolysis and releases of EF-Tu*GDP complex.
Peptide bond formation occurs, the nascent polypeptide chain is transferred from the P-site tRNA onto the aminoacyl moiety of the A-site tRNA. This reaction leaves an uncharged tRNA at the P-site and a peptidyl tRNA at the A-site.
To allow translation to proceed, translocation of the uncharged tRNA from the P-site to the E–site, and of peptidyl-tRNA from the A-site to the P-site, and presentation of the next codon of the mRNA in the A-site is required.
Translocation is mediated by the EF-G*GTP complex, which literally pushes the peptidyl-tRNA from the A-site to the P-site, upon GTP hydrolysis. The EF-G*GDP complex is then released, leaving the A-site empty for a new round of translation elongation.

Through repeated peptide bond formation the nascent protein chain increases in length, and is directed into the exit tunnel of the LSU.

1.3.3. Translation termination

The final phase of translation is termination. Normally, aa-tRNAs do not bind to the A-site containing a stop codon (UAA, UGA, or UAG). Instead, these stop codons are recognised by release factors (RF) which detache the polypeptide from the peptidyl-tRNA, thus from the ribosome.

The 70/ 80S ribosome is dissociated in a GTP dependent manner, by the ribosome release factor (RRF) and EF-G.

2. Ribosomes biogenesis and assembly.

In most pro- and eukaryotes the rRNA genes are transcribed as precursor rRNAs that are further matured by endo- and exonuleolytic cleavages, several chemicals modifications, and rRNA structural rearrangements. Despite rising knowledge, the full complement of enzymatic activities responsible for rRNA trimming/ cleavages has to be defined. In addition, ribosome biogenesis factors are transiently associated with nascent ribosomes and are suggested to be involved in the process of maturation/ assembly of ribosomes. Interestingly in archaeabacteria and eukaryotes, most rRNA modifications are depending on the formation of heteroduplexes of pre-rRNA with antisense small RNA. Moreover, in some cases these rRNA/ small RNA base pair interactions were suggested to mediate and/ or to stabilise rRNA structural rearrangements and further processing events.

Finally, in eukaryotes formation of cytoplasmic functional ribosomes requires intensive nuclear-cytoplasmic exchange.

Characteristic features of rRNA maturation and function of ribosome biogenesis factors will be presented in the following section with an emphasis on the yeast *S. cerevisiae*.
Noteworthy, r-proteins are also assembled onto the pre-rRNA during the course of ribosome biogenesis, however function and assembly of r-proteins will be described independently in section 3. of this introduction.

2.1. Ribosome components: genomic organisation and synthesis.

As shortly described above (introduction 1.2.1.), ribosome assembly requires the synthesis of more than 60 different macromolecules.
Furthermore, ribosome synthesis is a highly energy consuming process in cells. In **bacteria** about **10 000 ribosomes per cell** are found (Spirin, 2000) and can represent up to 50% of the bacterial dry mass (Tissieres et al., 1959), more than 50% of the total energy consumption is dedicated to ribosome production (Nierhaus, 2004). A similar situation exists in eukaryotes, i.e. **yeast growing cells contain about 200 000 cytoplasmic ribosomes**, however the amount of ribosomes synthesised varies and depends on the cell type and cellular growth conditions (see Warner, 1999).

Ribosome synthesis is thus the major metabolic pathway in actively dividing cells, which suggests that highly regulated mechanisms must have evolved to ensure the coordinated production of the different ribosome constituents according to cellular need (see for details Perry and Meyuhas, 1990; Planta, 1997; Nomura, 1999; Warner, 1999; Hornstein et al., 2001; Nomura, 2001; Nierhaus, 2004; Hamilton et al., 2006 and among other recent works Martin et al., 2004; Schawalder et al., 2004; Wade et al., 2004; Rudra et al., 2005; Hall et al., 2006; Zhao et al., 2006).

2.1.1. Genomic organisation and transcription of the rRNA genes in the cells.

Despite differences, some common principles of rRNA gene organisation in pro- and eukaryotes can be described (summarised in Fig. 5).

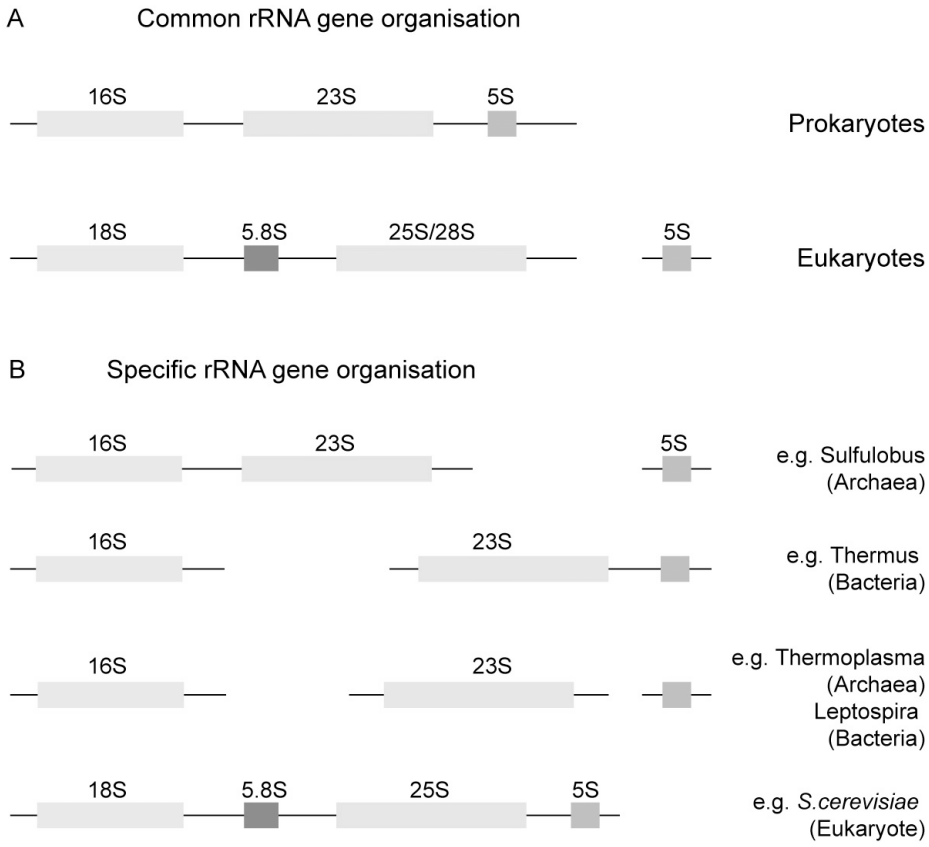


Figure 5: Various pattern of rRNA genes organisation. Adapted from Nierhaus, 2004.
(A) Common rRNA gene organisation in pro- (upper part) and eukaryotes (lower part).
(B) Some example of species specific rRNA gene organisation in pro- (upper part) and eukaryotes (lower part).

Most rRNA genes are organised as **operon-like structure** where the different rRNAs are transcribed into a common rRNA precursor containing the 16S, 23S and 5S rRNA and the 18S, 5.8S, 25/ 28S in most of the pro-and eukaryotes respectively. The different rRNAs are flanked and separated from each other by internal and external transcribed spacers (see Fig. 5A). This organisation might ensure stoichiometric rRNA production. However, in eukaryotes the 5S rRNA is transcribed independently. In addition, in some prokaryotes the rRNA are transcribed from independent genes (see Fig. 5B). Finally, it was shown in yeast that the SSU and LSU rRNA can be transcribed from independent genes (Liang and Fournier, 1997). Suggesting that this operon-like structure is not a prerequisite for production of functional ribosomes.

In eukaryotes, rRNA genes are often organised in tandemly repeated copies at one or a few chromosomal loci, whereas in bacteria the rRNA genes are scattered on the chromosome. The amount of rRNA gene repetition varies between organisms – from two in *M. jannashii* (Bult et al., 1996) to several thousands (for review see Hadjiolov, 1984-85). For example, *E. coli* possesses seven rRNA operons (Srivastava and Schlessinger, 1990), and the yeast *S. cerevisiae* about 150 tandemly repeated copies of the rRNA genes located on chromosome XII (see Goffeau et al., 1996; Nomura, 2001). It was shown is *S. cerevisiae* that the number of rRNA transcription units is dynamic and can vary depending on the environmental conditions (see Kobayashi et al., 1998; Klappenbach et al., 2000; Kobayashi et al., 2001).

In prokaryotes, all the cellular genes are transcribed with the help of a single RNA polymerase, whereas in eukaryotes there are three different kinds of RNA polymerases transcribing a different set of genes.

The RNA polymerase I (Pol I) is dedicated to the production of the rRNA contained in the operon-like structure (18S, 5.8S, 25/ 28S rRNA) (for review see Grummt, 2003; Moss, 2004; Moss et al., 2007), while the RNA polymerase III besides other cellular RNAs (e.g. tRNA) synthesises the 5S rRNA (for details and review see Geiduschek and Kassavetis, 2001).

Pol I dependent genes transcription occurs in a subnuclear compartment, the **nucleolus**. The nucleolus was first described by microscopy analysis in the early 19th century (see Wagner, 1835; Valentin, 1836 among others) and was found to be the location of rRNA synthesis (Perry, 1960; Errera et al., 1961; Perry and Errera, 1961; Brown and Gurdon, 1964). Furthermore, with the help of electron-microscopy techniques, the nucleolus can be subdivided into conserved morphological and functional ultrastructures: the **fibrillar centre or FC** containing the rDNA sequences, the **dense fibrillar component or DFC** containing

the Pol I machinery and early ribosome biogenesis factors, and the **granular component or GC** containing maturing pre-ribosomes (see Fig. 6 and Leger-Silvestre et al., 1999; Scheer and Hock, 1999). However, it should be noticed that these ultrastructural and functional definitions are still under debate (see Raska et al., 2004; Thiry and Lafontaine, 2005).

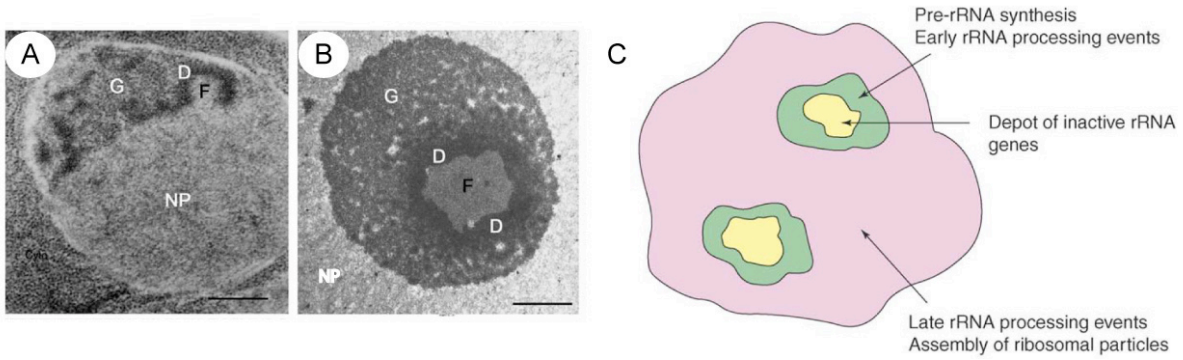


Figure 6: The nucleolus.
Electron micrographs of thin section nucleoli from (A) *S. cerevisiae* (Leger-Silvestre et al., 1999), (B) *X. laevis* (Scheer and Benavente, 1990) are shown.
Nucleoli show a tripartite organisation with a fibrillar center (FC indicated F in figure), dense fibrillar component (DFC indicated D in figure) and a granular component (GC indicated G in figure).
The scale bar in (A) and (B) represent 0.25 μm and 1 μm respectively. NP in (A) and (B) designates nucleoplasm.
(C) Schematic representation of nucleolar ultrastructure.
The respective ribosomal functions are ascribed to the FCs (yellow), DFCs (green) and granular component (GC) (pink) of the nucleolus. Reproduced from Raska et al., 2004.

Interestingly, the other cellular RNA polymerases are believed to be absent from this structure and expression of some RNA pol II dependent genes are silenced in the rDNA context (Bryk et al., 1997; Smith and Boeke, 1997). However, recent studies (review in Haeusler and Engelke, 2006, see also Thompson et al., 2003) indicate that the RNA Pol III dependent genes like the 5S rRNA and tRNA genes could be transcribed in the nucleolus or at its periphery. In addition, RNA Pol II dependent genes have been described to be expressed from the rDNA (Coelho et al., 2002; Kobayashi and Ganley, 2005). Further work need to be done in order to understand the relationship shared between all these machineries.

More recently, non-ribosomal domains were described in the nucleolus. Apparently the nucleolus plays unanticipated functions in the cells as e.g. among others the assembly of the signal recognition particle (for review see Pederson, 1998).

2.1.2. Genomic organisation and transcription of the r-protein genes.

In *E. coli* cells about half of the r-protein genes are clustered into four operons whereas the remaining r-proteins genes are scattered throughout the genome in operons containing 1–4 genes (review in Nierhaus, 2004).

In eukaryotes the r-proteins genes are scattered throughout the entire genome and are independently transcribed by RNA Pol II. Yeast *S. cerevisiae* r-protein gene promoters share common signatures: they often contain binding sites for the transcription regulators Rap1p and/ or Abf1p and T-rich sequences upstream of the transcription initiation start (Planta, 1997; Lascaris et al., 1999; Lascaris et al., 2000).

Noteworthy, in fungi and higher plants many r-protein genes exist in multiple copies and are coding for r-proteins isoform (see ribosomal protein gene database Nakao et al., 2004). In *S. cerevisiae* among the 78 r-proteins known so far 59 are encoded by duplicated genes, while the remaining once are encoded by single copy genes (see Kruiswijk and Planta, 1974; Mager et al., 1997; Nakao et al., 2004).

2.2. Maturation of ribosomal RNA.

2.2.1. Ribosomal RNA modifications in eubacteria and eukaryotes.

Nucleoside modifications of rRNA were already reported almost 50 years ago (Littlefield and Dunn, 1958b; Littlefield and Dunn, 1958a; Smith and Dunn, 1959). In all organisms, many nucleotides in the mature rRNA undergo covalent modifications. These modifications occur on the pre-rRNA during ribosome synthesis and are essentially of three types: **base methylation**; methylation of the 2'-hydroxyl group of sugar residues (**2'-O-methylation**); and conversion of uridine residues to **pseudouridine (Ψ)** by base rotation. Interestingly, the number of rRNA modifications increases from prokaryotes to eukaryotes (see Fig. 7). However, although these modifications are essentially clustered in the ribosome active centres, the nucleotides that are modified are not highly conserved.

	Eubacteria	Archaea	Eukaryotes	
	E.coli	*S.solfataricus*	*S.cerevisiae*	Human
Ψ sites	10	9	43	91
2´-O-methyl sites	4	67	55	106

Figure 7: The main rRNA modifications in pro- and eukaryotes.

Adapted from Lafontaine and Tollervey, 1998. The amount and types of the main rRNA modifications in representative organisms are indicated.

2.2.1.1. rRNA modifications in eubacteria.

For instance in eubacteria, rRNA modifications were described to be protein dependent. Different modifying proteins recognise the sequence and/ or structure of their target site directly. Apparently, sequence and (stable) structural informations are important since *in vitro* modification assays are strongly influenced by the substrate used (naked rRNA, and or by the composition of fully or partially assembled ribosomal subunit) (see for example Weitzmann et al., 1991; Lesnyak et al., 2006; Lesnyak et al., 2007). Moreover, most of these enzymes can modify closely related sequences or structures present in different RNA species, like tRNA (see for example Wrzesinski et al., 1995).

Despite rising knowledge about localisation and chemical nature of the rRNA modifications, some of the modifying enzymes still have to be identified (see for summary and recent identification report Rozenski et al., 1999; McCloskey and Rozenski, 2005; Andersen and Douthwaite, 2006; Lesnyak et al., 2006; Sergiev et al., 2006; Lesnyak et al., 2007).

2.2.1.2. rRNA modifications in archaeabacteria and eukaryotes.

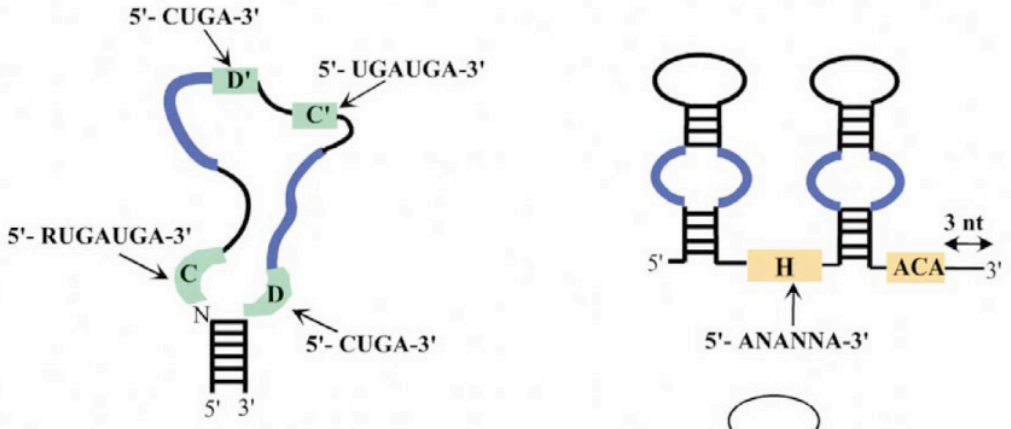
The most frequent rRNA modifications are **2'-O-methylation of ribose**, and uridine isomerization into **pseudouridines** (Ψ). Interestingly in both archaeabacteria and eukaryotes, rRNA modification sites are selected by formation of short heteroduplexes with antisense **s**mall (**n**ucle**o**lar) RNA (**snoRNA**). These rRNA/ snoRNA base pair interactions are necessary for rRNA processing and/ or rRNA modification events.

Less abundant are the base methylations that are, as far as it is known, dependent on protein-specific enzymes rather than snoRNP.

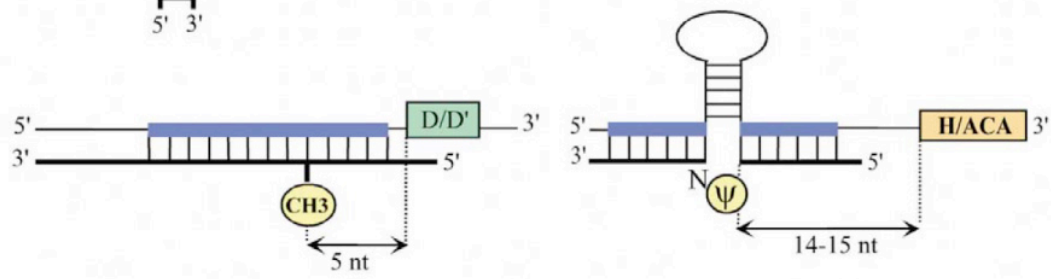
There are essentially two families of snoRNAs, the box **C/D** involved in sugar 2'-O-methylation, and the box **H/ACA** involved in (Ψ) formation.

Box C/D snoRNAs consist of a stem-loop structure with boxes C (UGAUGA) and D (CUGA) flanking a terminal helix; these boxes are sometimes duplicated (C' and D' box). Box H/ACA snoRNAs consist of two consecutive hairpin structures bridged by a conserved box H or hinge motif (ANANNA where N is any residue), and a triplet ACA always located 3 nucleotides upstream of the RNA 3' end (see Fig. 8A Lafontaine and Tollervey, 1998; Weinstein and Steitz, 1999).

A



B



C

	C/D snoRNPs		H/ACA snoRNPs	
	Eukarya	**Archaea**	**Eukarya**	**Archaea**
Core proteins	Nop1p (Fibrillarin)	Fibrillarin	Cbf5p (Dyskerin)	Cbf5
	Nop56p (p62)	Nop56 /Nop58	Gar1p	Gar1
	Nop58p		Nop10p	Nop10
	snu13p (15.5 kDa)	rpL7Ae	Nhp2p	rpL7Ae
Transiently-associated proteins	Srp40p (Nopp140)		Srp40p (Nopp140p)	
	SMN		SMN	
	p50 (rvb2)	p50/p55		
	p55 (rvb1)			
RNA targets	rRNAs, snRNAs	rRNAs	rRNA, snRNAs	
	mRNAs (?)	tRNAs	SL RNA (trypanosome)	rRNAs
			other RNAs ?	

Figure 8: Families of modification guide snoRNP. Reproduced from Bachellerie et al., 2002.
(A) Schematic secondary structures of the C/D and H/ACA classes of eukaryotic snoRNAs, with indication of their conserved box motifs (marked as green and orange boxes, respectively) and sequence tracts complementary to the cognate RNA target, also termed antisense elements (thick blue lines). The nucleotide targeted for modification is denoted by a yellow circle. For C/D snoRNAs (left), the 5'-3' terminal stem allowing the formation of the box C/D structural motif is represented. (B) Canonical structure of each type of guide RNA duplex. (C) Sets of associated proteins and categories of cellular RNA targets identified so far. Archaeal homologs of the two sets of snoRNP proteins are indicated.

At each site of modification, a duplex is formed by Watson-Crick base-pair interaction between a specific snoRNA and the RNA substrate. The snoRNA/ pre-rRNA hybrid precisely positions the residue to be modified on the substrate with respect to the conserved boxes of the snoRNA (see Fig. 8B and Cavaille et al., 1996; Kiss-Laszlo et al., 1996; Ganot et al.,

1997a; Ganot et al., 1997b; Ni et al., 1997; Kiss-Laszlo et al., 1998; Lafontaine and Tollervey, 1998; Weinstein and Steitz, 1999; Decatur and Fournier, 2003).

In eukaryotes, the snoRNAs are associated with a limited set of conserved core proteins specific for the C/D and H/ACA box. In archaeabacteria one of the core components is common to the C/D and H/ACA box, but is not conserved as a core component in eukaryotes snoRNP (see Fig. 8C). Apparently, for each type of snoRNP, one of the conserved core proteins Nop1p and Cbf5p (*S. cerevisiae* nomenclature) mediates the methyltransferase and the pseudouridine synthase activities respectively.

The snoRNAs and their associated proteins are all concentrated in the nucleolus, in the DFC, suggesting that rRNA modifications occur early in the course of ribosome biogenesis (Lischwe et al., 1985; Ochs et al., 1985; Henras et al., 1998). However, it is not clear how the modification process is temporally regulated during ribosome assembly. Early works in mammals and in yeast suggest that modifications occur co-transcriptionaly and early post-transcriptionaly respectively (see Warner et al., 1972; Udem and Warner, 1973 references and discussion therein). One can speculate, from the position of some modifications which are sometimes located within stem structures, that these modifications might occur prior to secondary structure formation (see Decatur and Fournier, 2002).
In any case, it is still unclear how rRNA maturation, modification, and r-protein assembly are coordinated.

Remarkably, few C/D or H/ACA box containing snoRNPs were described to be required for rRNA cleavages (see below). For instance in yeast, four snoRNPs are described to be required for rRNA maturation (Tollervey, 1987; Li et al., 1990; Hughes and Ares, 1991; Morrissey and Tollervey, 1993; Liang and Fournier, 1995; Dragon et al., 2002; Atzorn et al., 2004; Piekna-Przybylska et al., 2007). In fact, two of these snoRNAs, U3 (a box C/D) and snR30 (a box H/ACA), are not guiding any known modifications but are required for rRNA maturation (see below for details).

Extensive genome-wide bioinformatic analyses and/ or purification and cloning of small RNA from different organisms and tissues allowed to identify numerous small RNAs containing H/ACA or C/D boxes signature in all archaeabacteria and eukaryotes analysed (see e.g. Huttenhofer, 2006; Huttenhofer and Vogel, 2006). Almost all of these identified H/ACA or

C/D boxes containing small RNAs so far were described to have putative modification targets, some of which were experimentally confirmed.
Interestingly, H/ACA box motif and associated core proteins are components of the telomerase, which maintains the telomere length of chromosomes (Mitchell et al., 1999). In addition, some H/ACA or C/D boxes containing small RNAs were described to modify other RNA substrates like spliceosomal snRNA, tRNAs and putatively mRNA (see Fig. 8C and Bachellerie et al., 2002; Decatur and Fournier, 2003; Kiss, 2004).

In addition to snoRNPs, several putative base methyl-transferases have been described that, as far as it is known, do not involve snoRNAs for their function. A well-characterised example is the conserved protein Dim1p/ KsgAp (*S. cerevisiae*/ *E. coli* nomenclature respectively) involved in a conserved base dimethylation of SSU rRNA (see Lafontaine et al., 1994; Lafontaine et al., 1995; O'Farrell et al., 2006).

2.2.1.3. Function(s) of rRNA modifications.

"*The effect of nucleotide modification on rRNA is one of the oldest questions in RNA science and information(s) are still limited (Lane, 1998)*" (quoted from Decatur and Fournier, 2002).
The chemical properties of the modified nucleotides do not *a priori* point to specific functional roles, but several structural and thermodynamic effects have been proposed for RNA modifications (see for review Decatur and Fournier, 2002). In each case, the structural and thermodynamic effects depend on the structural context, and can extend beyond the site of modification.
Recently, positioning of the different rRNA modifications in *E. coli* and *S. cerevisiae* 3D ribosome structures have revealed that most modifications (~95% in *E. coli* and 60% in yeast) occur in functionally important regions. This include the peptidyl transferase centre, the A-, P- and E-sites of tRNA- and mRNA-binding, the polypeptide exit tunnel, and sites of subunit–subunit interaction (see for review Decatur and Fournier, 2002).
However so far, most if not all rRNA modifications have been found to be not essential for ribosome function (Ni et al., 1997; Green and Noller, 1999; Khaitovich et al., 1999; Lovgren and Wikstrom, 2001).
By contrast, strong negative effects on yeast growth occur when ( ) or 2'-O-methylation formation in rRNA is blocked on a global level, with point mutations in the putative ( )

synthase or methyl transferase enzymes (Tollervey et al., 1993; Zebarjadian et al., 1999). In prokaryotes, LSUs reconstituted with unmodified rRNA are active in peptide bond synthesis but have less activity than subunits reconstituted with natural rRNA (Green and Noller, 1999; Khaitovich et al., 1999)

Thus, it seems probable that most of the individual modifications contribute a small, non-essential benefit and that the full ensemble of modifications provides a large benefit. The evolution of parallel but different schemes to create the same types of modification suggests that these modifications must be beneficial (Badis et al., 2003; King et al., 2003).

2.2.2. Ribosomal RNA maturation in eubacteria and eukaryotes.

2.2.2.1. rRNA maturation in *E. coli*.

In *E. coli* the rRNA genes are transcribed into a 30S rRNA precursor containing the mature form of the 16S rRNA, a tRNA, 23S and 5S rRNA separated by extra and intragenic spacers (ETS and ITS respectively) (see Fig. 9). Several endo- and exonucleolytic reactions occur in order to liberate the different rRNA species from the pre-rRNA (see Fig. 9).

RNase III dependent maturation of 16S and 23S rRNA:

The 16S rRNA precursor is liberated from the 30S rRNA precursor through cleavages by the RNase III, leading to the formation of a pre-16S rRNA or 17S rRNA (Young and Steitz, 1978). Like for the pre-16S rRNA, a 23S rRNA precursor is liberated from the 30S rRNA precursor through cleavages by RNase III, leading to pre-23S rRNA formation (Bram et al., 1980; Sirdeshmukh et al., 1985; Sirdeshmukh and Schlessinger, 1985).

RNase III is not essential for cell growth, and recognises *in vivo* and *in vitro* double stranded RNA structures (Ginsburg and Steitz, 1975; Gegenheimer and Apirion, 1980), apparently without sequence specificity *in vitro* despite the fact that *in vivo* RNase III substrates seem to share a consensus sequence (Chelladurai et al., 1991).

Interestingly, RNase III is not only involved in pre-rRNA cleavage, but also cleaves some mRNAs (review in Nicholson, 1999; MacRae and Doudna, 2007)

In RNase III deficient strains pre-16S rRNA and pre-23S rRNA produced are slightly larger (Gegenheimer et al., 1977) presumably resulting of normal neighbourhood processing events

in tRNA, 5S rRNA and “non specific” cleavages. Surprisingly, in a RNase III deficient strain, mature 16S rRNA is produced with the same rate as in a wildtype strain (see Srivastava and Schlessinger, 1990). In contrast, RNase III deficient strains produce a slightly larger heterogeneous functional 23S rRNA (King et al., 1984).

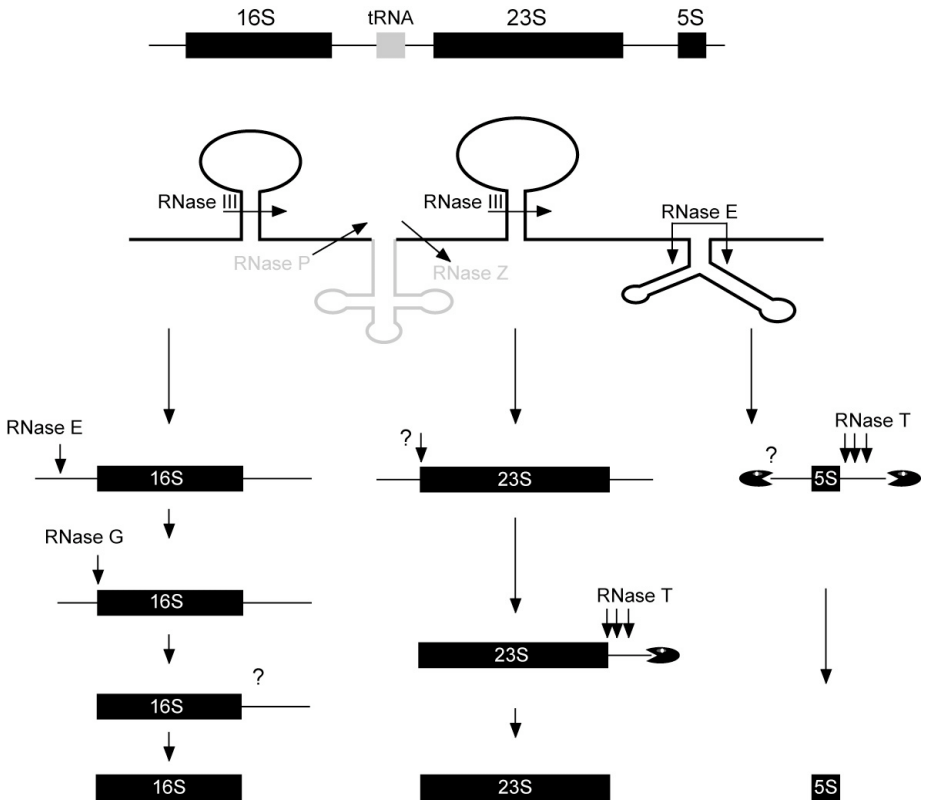


Figure 9: Ribosomal RNA processing in *E. coli*.
Adapted and updated from Nierhaus, 2004 and references in introduction 2.2.2.1.
Upper part shows the operon structure of the rRNA genes in *E. coli*. Below a cartoon of the operon secondary structure of the respective RNA is represented.
The cleavage sites are indicated with arrows and when known the RNase triggering the processing reaction is indicated.

Final 16S rRNA maturation:

After RNase III cleavage, 5’ and 3’ mature termini are formed by further cleavages.

Remarkably, in contrast to *in vitro* reconstituted RNase III dependent cleavage, *in vitro* reconstitution of the following maturation steps depends on the pre-formation of rRNA – r–protein complexes (Schlessinger, 1979).

Early work identified in a particular mutant strain an rRNA intermediate containing a 66□ucleotide extension at the 5’-16S rRNA terminus and a mature 3’ end. The 5’ end could

be further matured *in vitro* into 16S rRNA by incubation with a wildtype cell extract (Dahlberg et al., 1978). This suggested that 5'-16S rRNA terminus is probably matured in two steps. Recently, it was shown *in vivo* and *in vitro* that maturation of the 5'-16S rRNA terminus depends on two closely related enzymes: RNase E and RNase G (Li et al., 1999b; Lee et al., 2002).
Both enzymes are multifunctional enzymes involves in the metabolism of several cellular RNAs (Li et al., 1999b; Nicholson, 1999; Carpousis, 2002; Lee et al., 2002 and below).

The RNase E is essential for cell viability, but in the absence of RNase E formation of mature 16S rRNA still occurs with a slow rate. The RNase G is non essential for cell viability, and interestingly depletion of RNase G reduces the amount of 16S rRNA produced and leads to the accumulation of a 16S pre-rRNA containing a 66 nucleotides extension at the 5' terminus (Dahlberg et al., 1978; Li et al., 1999b). Depletion of both RNase E and G leads to a complete inhibition of 16S rRNA production and accumulation of a 17S rRNA species which is cleaved *in vitro* using purified RNase E at position +66 relative to the 5' end.

The activity responsible for the 16S rRNA 3' end maturation *in vitro* was isolated but so far not fully characterised (Hayes and Vasseur, 1976). Interestingly, it was suggested that extensions of pre-16S rRNA (17S rRNA) can potentially base pair (see Srivastava and Schlessinger, 1990). Furthermore, results by Li et al (Li et al., 1999b) indicate that 3' maturation depends to some degree on efficient maturation of the 5' end terminus. This suggests a possible inhibitory effect on 3' end processing by formation of a terminal stem like structure.

Final 23S rRNA maturation:
After RNase III cleavage, mature 5' and 3' termini are formed by further cleavages.
Early study suggests that 23S rRNA 5' end maturation is probably carried out by a so far unidentified endonuclease and requires conditions enabling protein synthesis (King et al., 1984; Srivastava and Schlessinger, 1988). Another report suggested that 23S rRNA 3' end digestion proceeds through exonucleolytic reactions (Sirdeshmukh et al., 1985; Sirdeshmukh and Schlessinger, 1985). Recently, analysis of maturation of rRNA in strains defective for different known RNases indicated that the RNase T is required for 23S rRNA 3' end maturation (Li et al., 1999a). RNase T is also involved in the maturation of various cellular RNAs (e.g. 5S rRNA, tRNA) (see for review Nicholson, 1999 and below).

A strain lacking the non-essential RNase T shows an extended 3'-23S rRNA terminus while the 5'-23S rRNA is normally processed, and a small amount of fully matured 23S rRNA is produced suggesting that (an)other RNase(s) can proceed with 5' end maturation, however with a poor efficiency. In addition, pre-23S rRNA containing particles isolated from RNase T deficient cells can be further matured *in vitro* by addition of purified RNase T (Li et al., 1999a).

In summary, RNase III dependent 23S rRNA intermediate formation is a prerequisite for final maturation of 23S rRNA and precedes maturation of 5' end 23S rRNA by an unknown endonuclease and 3' end formation involving mainly the exonuclease RNase T.

5S rRNA maturation:

The 5S rRNA is released from the pre-30S rRNA by the action of the endoribonuclease, RNase E (Misra and Apirion, 1979). The 5' residues are suggested to be removed by an exonucleolytic process (Roy et al., 1983), but the factor involved in this process is still unknown. The 3' residues are removed *in vitro* through action of the exonucleolytic activity of purified RNase T, and *in vivo* depletion of RNase T leads to the accumulation of functional ribosomes containing extra nucleotides at the 5S rRNA 3' end, meanwhile 5' processing occurs independently (Christiansen, 1988; Li and Deutscher, 1995).

2.2.2.2. rRNA maturation in archaeabacteria.

In archaeabacteria, information about the rRNA maturation pathway is fragmented. However, recent analysis in two representative species from the major archaeal kingdoms (*Archaeoglobus fulgidus* and *Sulfolobus solfataricus*, euryarchaeota and crenarchaeota respectively), suggests a general rRNA maturation pathway in archaeabacteria (see Fig. 10 Tang et al., 2002).

Apparently the 16S and 23S rRNA precursors form a secondary structure - found also in tRNA precursors in archaeabacteria – called bulge-helix-bulge or BHB (Kjems and Garrett, 1988; Lykke-Andersen et al., 1997). The BHB motif consists of two 3 nucleotides bulges on opposite strands of the RNA, separated by a 4 nucleotides helix, this motif is cleaved by a so called splicing endonuclease (Tang et al., 2002). Cleavages are followed by ligation of the pre-rRNA extremities with the help of an unknown ligase activity, thus releasing circular pre-16S and pre-23S rRNA, and the ligation product of the 5' ETS and 3' ETS.

The pre–rRNAs are finally further matured through not yet determined endo and/ or exonucleolytic cleavages (Tang et al., 2002; Dennis and Omer, 2005).

Interestingly, secondary structure analysis, and chemical probing indicate that the 5' and 3' ETS of both pre-16S and pre-23S rRNA form a C/D box snoRNA like structures which are also able to bind archaeal snoRNP-like core protein components (Tang et al., 2002). However, it needs to be determined, whether these RNA structures have any relevant function(s) *in vivo*.

In addition, it was shown in *S. solfataricus* that the 5' ETS of the pre-16S rRNA can be processed by endonucleolytic reaction *in vivo* and *in vitro* at two main sites upstream of the BHB motif (Durovic and Dennis, 1994; Russell et al., 1999; Ciammaruconi and Londei, 2001). Remarkably the neighbouring sequences of the most 5' cleavage site show sequence homology between crenarchaeota and eukaryotes, and is analogous to the eukaryotes A0 rRNA processing site (Ciammaruconi and Londei, 2001).

Finally, 5S rRNA maturation in archaeabacteria is still uncharacterised.

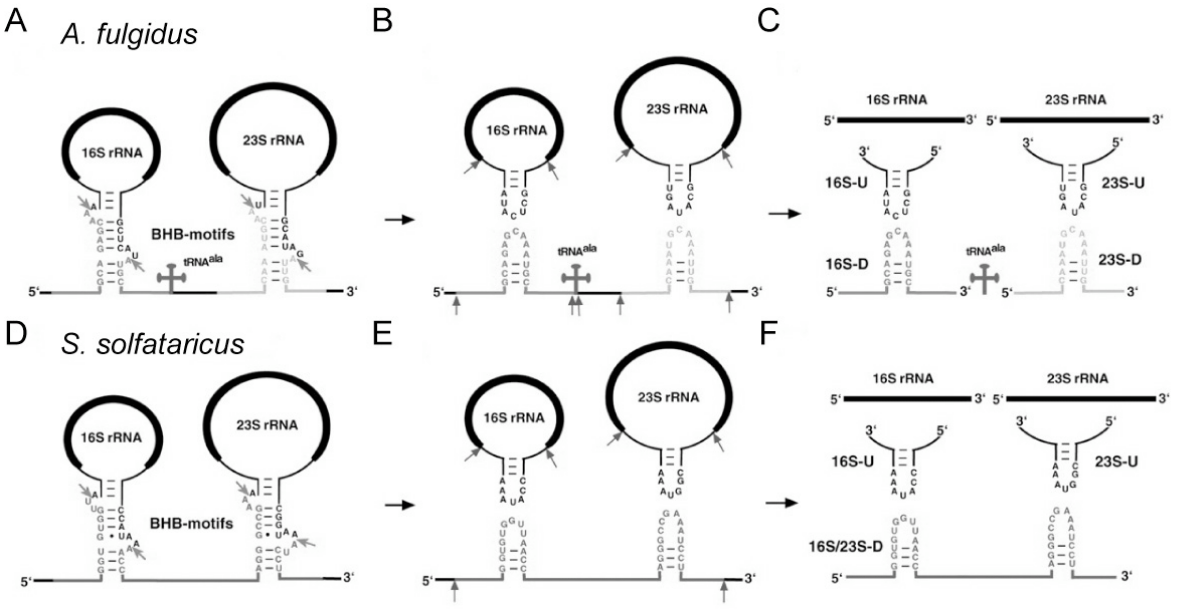


Figure 10: Proposed pre-rRNA processing pathway in archaeabacteria. Reproduced from Tang et al., 2002.

Ligated RNA species from processing stems of the ribosomal operon of *A. fulgidus* (A–C) and *S. solfataricus* (D-F). Proposed pre-rRNA processing pathway in *A. fulgidus* (A-C) and *S. solfataricus* (D-F). Mature rRNAs are represented by thick black lines, precursor sequences by thin lines. BHB motifs are shown; the cleavage sites within BHB motifs are indicated by arrow (A and D). Further processing steps of ligated RNA species are shown by arrows (B and E).

2.2.2.3. Ribosomal RNA maturation in eukaryotes.

In eukaryotes, rRNA maturation is best studied in the yeast *S. cerevisiae*, and seems to be well conserved among eukaryotes (among others Hadjiolova et al., 1993; Gerbi et al., 2003; Rouquette et al., 2005; Choesmel et al., 2006). Despite numerous efforts, many of the endo- and exo-nucleases responsible for rRNA maturation are unknown. Most of the rRNA maturation steps could not be reconstituted *in vitro* making the identification of most of the enzymatic activities responsible for rRNA processing difficult.

So far, most of our knowledge is based on *in vivo* loss of function analysis of many factors that indicates their requirement for specific cleavages. However, their exact function in the maturation process remains to be determined.

The current view of rRNA processing in *S. cerevisiae* is summarised in Fig. 11.

A more detailed view about the rRNA maturation *cis*- and *trans*-acting elements is described below.

Co-transcriptional and early rRNA processing events.

The longest detectable pre-rRNA transcript in yeast, the 35S pre-rRNA, extends from the transcription initiation site to a position several nucleotides beyond the 3' end of mature 25S rRNA (see Fig. 11). The actual transcription termination site is predicted to be approximately 200 nucleotides further downstream of the 25S rRNA 3' end, but these intermediates are undetectable in wildtype cells, this and recent work suggest that 35S pre-rRNA is probably released co-transcriptionaly (see Henras et al., 2004).

Release of 35S pre-rRNA is carried out by the endoribonuclease Rnt1p (Kufel et al., 1999), the yeast homologue of bacterial RNase III (King et al., 1984). Rnt1p-dependent cleavage of the 3' ETS is observed *in vivo* at positions +14 and +49 with respect to the mature 3' end of the 25S rRNA (Kufel et al., 1999), suggesting that the 35S pre-RNA actually extend to +14. The 3' ETS cleavage sites lie on both sides of a 5'-AGNN-3' tetra-loop structure which is a strong determinant for Rnt1p-binding and cleavage positioning on the nascent pre-rRNA (Chanfreau et al., 2000; Nagel and Ares, 2000; Lamontagne et al., 2003; Lamontagne and Elela, 2004; Leulliot et al., 2004; Wu et al., 2004).

Like RNase III, Rnt1p is a multifunctional enzyme involved in the metabolism of many cellular RNAs (reviewed in Lamontagne et al., 2001; MacRae and Doudna, 2007).

In strains lacking Rnt1p or carrying mutations in the stem-loop structure in the 3' ETS, cleavage does not occur and 3' extended pre-rRNA species are formed (Kufel et al., 1999). The most abundant 3' extended pre-rRNA in a *rnt1* mutant extends to a position near the major Pol I transcription stop at position +210 (van der Sande et al., 1989; Kufel et al., 1999). With or without Rnt1p dependent cleavage, pre-35S rRNA undergoes a serie of maturation events that are described below.

Recently, electron microscopy visualisation of the transcribed rRNA genes using Miller's chromatin spreading technique (Miller and Beatty, 1969) in wildtype and mutant yeast cells, suggested that co-transcriptional cleavage in the ITS1 can occur with a relative high frequency (see Osheim et al., 2004). These results suggest that - like in *Dictyostelium* (Grainger and Maizels, 1980), *E. coli* (Hofmann and Miller, 1977), and unlike most other eukaryotes analysed (Osheim et al., 2004) - separation of SSU and LSU rRNA precursors in yeast can happen by early co-transcriptional cleavage events.

Final 18S rRNA maturation steps.

Pre-18S rRNA is released from the 35S rRNA precursor as a 20S rRNA precursor through cleavages at sites A0, A1 and A2 (see Fig. 11). Alternatively, pre-18S rRNA is released as a 23S rRNA precursor through cleavage at site A3 (see Fig. 11). Whether the 23S pre-rRNA is further processed normally to produce functional 18S rRNA is still under debate, since this rRNA was for a long time considered to be an aberrant rRNA species (see among others Venema and Tollervey, 1995; Venema and Tollervey, 1999).

The 35S and probably the 23S pre-rRNAs are processed into 20S pre-rRNA through endonucleolytic cleavages at sites A0, A1 and A2, which are strongly coupled (Kressler et al., 1999).

The A0 processing site in the 5' ETS was identified via primer extension and is located 90 nucleotides upstream of the 5' end of mature 18S rRNA (Hughes and Ares, 1991; Beltrame et al., 1994). The A1 processing site corresponds to the 5' mature end of 18S rRNA (see Fig. 11) and the A2 processing site is positioned 214 nucleotides downstream of the 3' end of the mature 18S rRNA (van Nues et al., 1994; van Nues et al., 1995).

Mutational analyses of *cis*-acting elements involved in the A1 site cleavage suggest that two mechanisms determine cleavage at site A1. One mechanism involves recognition of evolutionarily conserved nucleotides immediately upstream of A1 in the 5' ETS, whereas a

second mechanism positions the cleavage site at a fixed distance from a conserved stem-loop structure within the mature 18S rRNA 5' end (Venema et al., 1995; Sharma et al., 1999). Surprisingly, substitution of the evolutionarily conserved nucleotides across the A1 site abolished processing at A0 without any significant effect on A1 cleavage, 18S rRNA synthesis, or cell viability (Venema et al., 1995) suggesting that the A0 cleavage is somehow not essential for rRNA maturation. Furthermore secondary structure prediction of the 5' ETS region suggests that the A0 and A1 cleavage sites are in close proximity at opposite sides and near the base of an extended stem-loop structure (Yeh and Lee, 1992; Intine et al., 1999).

The *cis*-acting elements involved in the A0 site cleavage are still unclear. Despite the fact that the sequence of the A0 cleavage site is evolutionarily conserved, mutations across this cleavage site do not affect neither the efficiency nor the cleavage position (Venema et al., 1995).

Interestingly, a similar cleavage recognition mechanism as described for the A1 site, appears to act at site A2 in ITS1. Cleavage at site A2 is also positioned with respect to conserved sequences across and flanking the A2 site and to a secondary rRNA structure 3' of the cleavage site (Allmang et al., 1996). Remarkably, it was demonstrated, when expressing the pre-18S rRNA independently from LSU pre-rRNA using a split operon, that in this condition the A2 site is not required neither for SSU nor LSU rRNA formation (Liang and Fournier, 1997). This result strongly suggests that the main A2 site function is to separate the SSU and the LSU maturation pathways.

In addition to the different cleavage sites, other *cis* elements promoting SSU assembly and maturation have been described. In fact, it was demonstrated that direct base pairing between the pre-rRNA and some snoRNAs is required for cleavage reaction. In yeast and vertebrate one of the best studied snoRNA is the box C/D snoRNA U3 (Beltrame and Tollervey, 1992; Beltrame et al., 1994; Beltrame and Tollervey, 1995; Hughes, 1996; Gerbi et al., 2003).

The U3 snoRNA is required for A0, A1 and A2 processing, and binds to the 5' ETS, 230 nucleotides upstream of site A1 and to the mature 18S rRNA. Interestingly, mutating in *cis* the pre-rRNA elements interacting with U3, mimic the phenotype observed in *trans* after U3 depletion (Hughes and Ares, 1991; Beltrame and Tollervey, 1992; Beltrame et al., 1994; Beltrame and Tollervey, 1995; Hughes, 1996).

Based on the observed interaction of U3 with the pre-rRNA an attractive model of pre-rRNA folding was suggested, where the 5' and 3' rRNA sequences would be brought into close contact inducing a circle-like structure of the pre-18S rRNA as described in prokaryotes

(Hughes, 1996; Gerbi et al., 2003). Noteworthy, (1) based on the knowledge on rRNA processing in *E. coli*, (2) in combination with predicted base pairing between the 5' ETS and the ITS1, and (3) the tight linkage between the processing events at A0, A1 in the 5' ETS and A2 in the ITS1, a similar looping model was already suggested in the beginning of the 1980s (see Veldman et al., 1981).

The 20S pre-rRNA is exported from the nucle(ol)us (Udem and Warner, 1973), and subsequently processed at site D, the 3' end of mature 18S rRNA (see Fig. 11).

Detection of full-length D–A2 fragment indicates that this step, like the others in 18S rRNA synthesis, is endonucleolytic, although the responsible enzyme remains to be identified (Stevens et al., 1991). The *cis* element(s) required for accurate processing at site D are still unclear. Apparently, the ITS1 region from several other, more or less closely related, yeast species can functionally replace the *S. cerevisiae* counterpart despite large differences in size and sequence (van Nues et al., 1994). However, using a system expressing separately the pre-18S rRNA and pre-5.8S/ 25S rRNA, it has been suggested that only 44 nucleotides downstream the site D are sufficient for proper 18S rRNA maturation (see Liang and Fournier, 1997). This suggests that most of the ITS1 region is dispensable for 18S rRNA. More recently, mutational analysis suggested that the *cis* elements required for site D cleavage are located on both sides in the immediate vicinity of the cleavage site (van Beekvelt et al., 2001b).

Secondary rRNA structure prediction indicates that site D is located at the apical end of a hairpin formed by base pairing between the 3' end of 18S rRNA and the 5' end of ITS1 (Yeh et al., 1990). However this structure in the upper region of the helix-containing site D is not important for processing at site D. This rRNA secondary structure is neither required for production of functional 40S ribosomal subunits (van Beekvelt et al., 2001b).

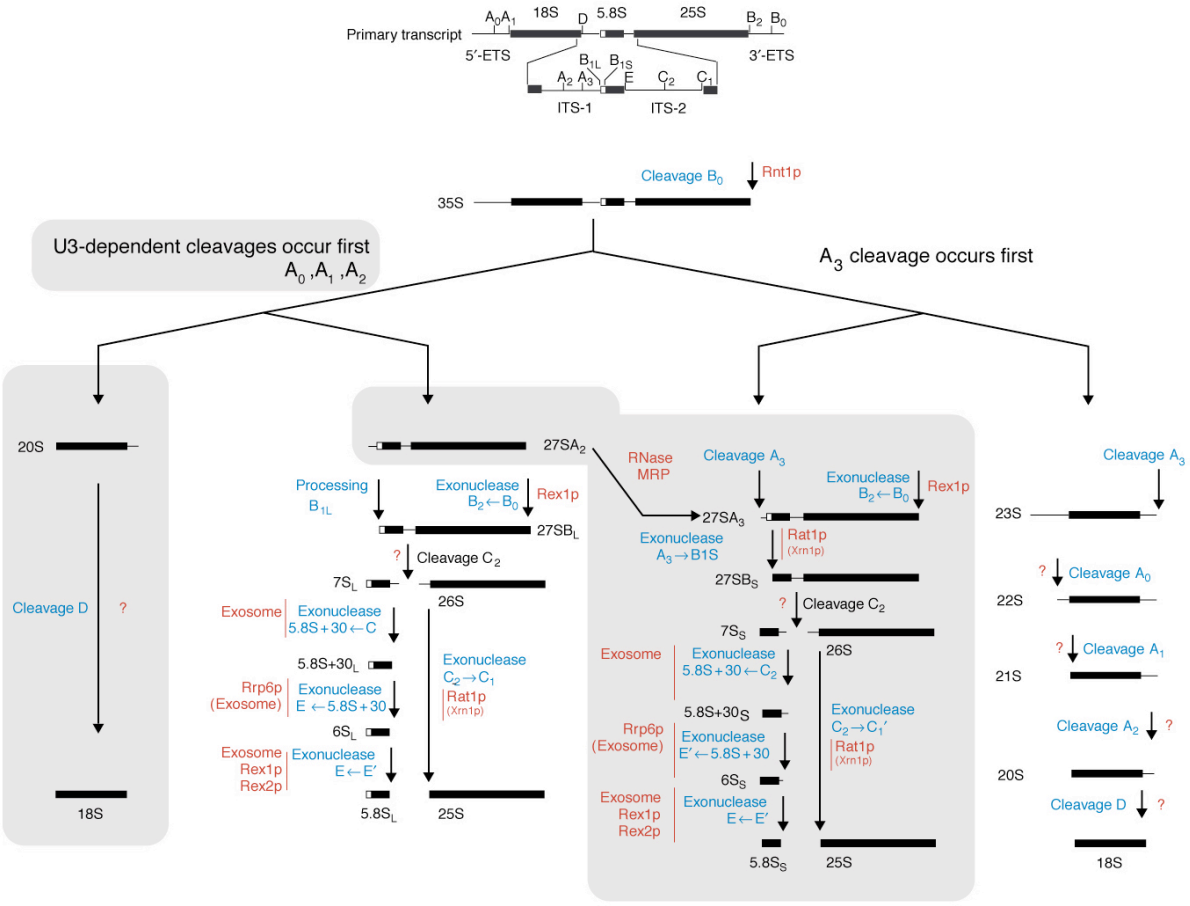


Figure 11: Pre-rRNA processing pathway in *Saccharomyces cerevisiae*. Adapted from Fatica and Tollervey, 2002; Fromont-Racine et al., 2003; Gallagher et al., 2004.

Upper panel: structure of the pre-rRNA and locations of processing sites. In the pre-rRNA, the regions encoding the 18S, 5.8S and 25S rRNAs are flanked by the 5´ and 3´ external transcribed spacers (5´-ETS and 3 ´-ETS) and separated by internal transcribed sequences 1 and 2 (ITS-1 and ITS-2). Lower panel: pre-rRNA processing in *Saccharomyces cerevisiae*. Known processing enzymes are indicated in red. Unidentified enzymes are indicated as question marks. The gray background indicates the major rRNA processing pathways. See introduction 2.2.2.3. for details.

Final 25S & 5.8S rRNA maturation steps.

In contrast to the 18S rRNA maturation pathway, maturation of 25S & 5.8S rRNA is more complex, and requires both endonuclease cleavages and exonuclease digestions. In addition several enzymatic activities involved in the 5.8S/ 25S rRNA maturation pathway have been characterised.

After cleavage of the 35S pre-rRNA at site A2 or A3 the 27SA2 or 27SA3 rRNA precursors are released.

The 27SA2 rRNA precursors are matured using two alternative pathways: a major pathway (85%) and a minor pathway (15%) (reviewed in Venema and Tollervey, 1995; Venema and Tollervey, 1999).

Using the major pathway the 27SA2 rRNA is cleaved at site A3 by an RNP complex the RNase MRP (Shuai and Warner, 1991; Lindahl et al., 1992; Chu et al., 1994; Schmitt and Clayton, 1994; Chamberlain et al., 1998). The RNase MRP is an essential eukaryote specific endonuclease related to bacterial RNase P, which is required for rRNA processing, but also in the metabolism of other RNAs (see Morrissey and Tollervey, 1995; Walker and Engelke, 2006). Remarkably, cleavage at site A3 could be reproduced *in vitro* using purified components from yeast (Lygerou et al., 1996). Cleavage at A3 can be inhibited by deletion of 10 nucleotides across the A3 sequence (Henry et al., 1994). Interestingly in this condition maturation of 27SA2 pre-rRNA proceeds through the minor pathway (Henry et al., 1994). In fact, site A3 acts as an entry site for the 5'→ 3' exonucleases Xrn1p and Rat1p which generate the 5.8S rRNA 5' end, further named $5.8S_S$ (Henry et al., 1994). Xrn1p and Rat1p are two homologous proteins, which show *in vitro* processive 5'→ 3' exoribonuclease activity and are also involved in RNA metabolism (see among others Stevens et al., 1991; Amberg et al., 1992; Beelman and Parker, 1995; Johnson, 1997; Petfalski et al., 1998).
Meanwhile, Rex1p dependent 3'→ 5' exonucleolytic digestion occurs from B0→ B2 and generates the 3' mature 25S extremity (Kempers-Veenstra et al., 1986). These two parallel reactions generate the $27SB_S$ (see Fig. 11). Rex1p is closely related to the prokaryotic RNase□D and is involved in the trimming of different RNA substrates (Kempers-Veenstra et al., 1986; van Hoof et al., 2000)

Interestingly, deletion analysis of the 3' ETS region indicates that the stem loop structure recognised by Rnt1p is both necessary and sufficient for complete processing in the 3' ETS (Allmang and Tollervey, 1998). Finally, loss of the hairpin structure in the 3' ETS strongly inhibits processing at site A3 in the ITS1, and shifts the LSU biogenesis towards the minor pathway, suggesting that ITS1 and 3' ETS cleavages are coordinated (Allmang and Tollervey, 1998). Again agreeing with the looping model proposed in the beginning of the 1980s by Veldman et al., (Veldman et al., 1981). Interestingly in higher eukaryotes, it was suggested that such a looping of pre-rRNA would be enabled by base pairing of the U8 snoRNA with the pre-rRNA (Peculis, 1997). In yeast, U8 snoRNA does not exist, however it is possible that an U8-like activity would stabilise base pair interaction, and further processing. This attractive model needs to be further demonstrated.

Using the minor pathway the 27SA2 rRNA is cleaved in 5' at site $B1_L$ by an unknown endonuclease (review in Venema and Tollervey, 1995; Venema and Tollervey, 1999; Faber et al., 2006) and in 3' at site B2 by 3'→5' exonucleolytic digestion (see earlier and Kempers-Veenstra et al., 1986) generating $27SB_L$.
Despite sequence conservation at the $B1_L$ site mutational analysis reveals that this sequence is not required for processing at $B1_L$ site (Faber et al., 2006). So far neither *cis*-acting elements nor enzymatic activities directing this cleavage, have been described.

Further on, the same pathway is used to process both $27SB_S$ and $27SB_L$. First 5.8S and 25S rRNA precursors are separated into $7S_{S\ or\ L}$ and 26S rRNA respectively through cleavage at site C2 in the ITS2 by an uncharacterised activity (reviewed in Venema and Tollervey, 1995; Venema and Tollervey, 1999).

The pre-26S rRNA is matured into 25S rRNA, mainly by Rat1p or by Xrn1p (however with less efficiency) dependent 5'→3' exonucleolytic digestion (Geerlings et al., 2000).

The final maturation of the pre-$7S_{S\ or\ L}$ rRNA 3' end is believed to occur through multiple exonucleolytic digestion steps, since several intermediates are detectable.
First 3'→5' exoribonuclease digestion occurs from the C2 site to 30 nucleotides downstream of the 3' end, generating the 5.8S+30 rRNA. Formation of the 5.8S+30 rRNA is mediated by a large protein complex - the exosome (Mitchell et al., 1996; Mitchell et al., 1997) – containing several components all of which have either been shown to have 3'→5' exoribonuclease activity *in vitro* or are predicted to have it based on sequence homology. Some of the exosome components, in addition to their 3'→5' exoribonuclease activity, are homologous to *E. coli* RNases (Mitchell et al., 1997; Venema and Tollervey, 1999). Second, further 3'→5' exoribonuclease digestion mediated by the exosome – apparently through action of sub-components Rrp6p and Rrp47p - process the 5.8S+30 rRNA into 6S rRNA (3'-end with 8 nt extended) (Briggs et al., 1998; Mitchell et al., 2003). Third, 3 nucleotides at the 3' end 5.8S rRNA are further released by the action of the exosome and two homologous proteins Rex1p and Rex2p which share also homology with the bacterial RNase D (van Hoof et al., 2000). Finally, the last 4 nucleotides are released by the non-essential factor Ngl2p, probably by endonucleolytic cleavage, producing mature 5.8S rRNA (Faber et al., 2002).

Interestingly, the exosome is involved in the degradation of mRNA (Houseley et al., 2006). Recent work, in the yeast *S. cerevisiae* suggest that aberrant rRNA precursors are recognised (by an unknown mechanism), marked by poly-adenylation, and further degraded by a nuclear specific form of the exosome (see Kuai et al., 2004; LaCava et al., 2005; Houseley et al., 2006).

It is difficult to determine the *cis*-acting elements that are required for processing within the ITS2, since in contrast to the ITS1, the ITS2 is strictly required for proper maturation of both 5.8S and 25S rRNA. In fact, complete or partial deletions or mutations within ITS2, or replacement of ITS2 in *S. cerevisiae* with counterparts from other species, or independent expression of the LSU cistrons, fail to produce mature 5.8S and 25S rRNA. (van der Sande et al., 1992; van Nues et al., 1995; Liang and Fournier, 1997; Peculis and Greer, 1998; Cote and Peculis, 2001; Cote et al., 2002).
In addition, two alternative secondary structure models exist for *S. cerevisiae* ITS2. Chemical and enzymatic structure probing and phylogenetic comparisons resulted in one structure model (Yeh and Lee, 1990) referred as the "hairpin model" and an alternate folded structure model was proposed (Joseph et al., 1999; Michot et al., 1999), referred as the "ring model". Mutation analysis indicates that mutually exclusive structural elements of the ring and hairpin models may play distinct roles in processing and suggests that a transition between the respective conformations may be important for processing (Cote et al., 2002). Accordingly a dynamic conformational model for ITS2 was proposed: initial formation of the "ring structure" may be required for essential, early events in processing complex assembly and may be followed by an induced transition to the "hairpin structure" that facilitates subsequent processing events (Cote et al., 2002).

5S rRNA maturation.

The 5S rRNA genes are transcribed as a 5S pre-rRNA. The transcription initiation site corresponds to the mature 5S rRNA. The 3' end is extended by 7 to 13 nucleotides that are digested by a 3'→5' exonuclease activity (Piper et al., 1983; van Hoof et al., 2000). Complete 3'→5' digestion of the 5S rRNA is apparently blocked by a secondary RNA structure formed at the 3' end of mature 5S rRNA (see Lee and Nazar, 1997; Lee and Nazar, 2003).

2.3. Transient interactions of ribosome biogenesis factors with pre-ribosomes and their function in eukaryotic ribosome biogenesis.

In the early 1970s, with the help of metabolic labelling experiments, cell fractionation and sucrose-gradient analysis, a basic model of eukaryotic ribosome assembly was defined and remains the core of today's ribosome assembly knowledge. These experiments revealed that an early 90S pre-ribosome particle containing 35S pre-rRNA is formed and subsequently separated into 43S and 66S particles containing SSU and LSU rRNA precursors respectively. The 43S pre-ribosomes are then exported to the cytoplasm and matured into 40S SSU, whereas maturation of the 66S particles continues in the nucleus prior to export (see Fig. 12). Interestingly, these analyses were also indicating that the pre-ribosomal particles contain a higher protein to RNA ratio compared to the mature ribosomes. This suggested the existence of transiently associated protein factors.

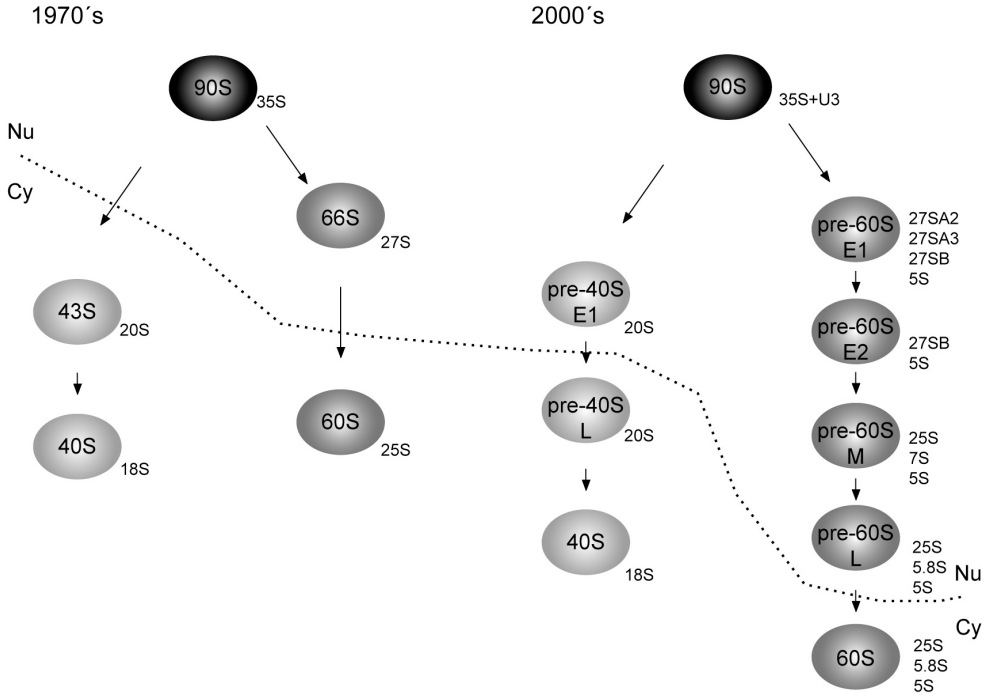


Figure 12: Evolution of the ribosomal assembly pathway.
Modified from Nierhaus, 2004. See also introduction 2.3. for details.
Pre-ribosome have tentatively been ordered, based on their protein and rRNA content, and respective sub–cellular localisation.
The left panel shows the ribosomal assembly pathway as was demonstrated in the 1970´s.
The right panel shows the ribosomal assembly pathway as it was demonstrated in the beginning of 2000´s. E: Early, M: Middle, L: Late, Nu: Nucleoplasm, Cy: Cytoplasm.

With the help of powerful genetic approaches in yeast, factors involved in ribosome biogenesis could be identified. However these genetic approaches were far from being exhaustive in describing the full set of factors required for a given step of ribosome maturation.

Only recently, the tools required for the isolation of pre-ribosomal particles and subsequent identification of the associated proteins have become available. **Tandem affinity purification (TAP)** of epitope-tagged proteins allowing the isolation of pre-ribosomal complexes under physiological conditions (Rigaut et al., 1999) in combination with mass spectrometry identification of the components became a powerful method to analyse the association of ribosome biogenesis factors with pre-ribosomes. As a result, **more than 120 non-ribosomal proteins** were found in association with pre-ribosomal particles in yeast (see Fig. 13).

According to their sub-cellular localisation, and their pre-rRNA and/ or protein interaction pattern, these factors were placed in a relatively precise eukaryotes ribosome biogenesis factor assembly map (see Fig. 12-13, and Fromont-Racine et al., 2003; Milkereit et al., 2003a; Hage and Tollervey, 2004).

In addition, systematic *in vivo* depletion and/ or mutant analysis of most of these non-ribosomal proteins factors demonstrated their requirement at different steps of the rRNA maturation pathway, and/ or for pre-ribosomal particles export.
However, the exact molecular function for most of these factors is unclear. Among these factors, few were predicted or were shown to be **ATP dependent RNA helicases, ATPases, GTPases,** and **Kinases**. They are suggested to facilitate ribosome assembly and/ or rRNA folding, or to unwind the snoRNA-pre-RNA duplex (see for review Fromont-Racine et al., 2003; Hage and Tollervey, 2004, among recent work Karbstein et al., 2005; Bernstein et al., 2006; Granneman et al., 2006, and following paragraphs).
Finally, these biochemical approaches were focusing on the identification, of stoichiometrically co-purifying factors, neglecting most of the sub-stoichiometrically co-purifying factors as a result many of the enzymes known to trigger rRNA maturation failed to be identified (Hage and Tollervey, 2004).

A more detail view of these results will be given in the following part of this introduction.

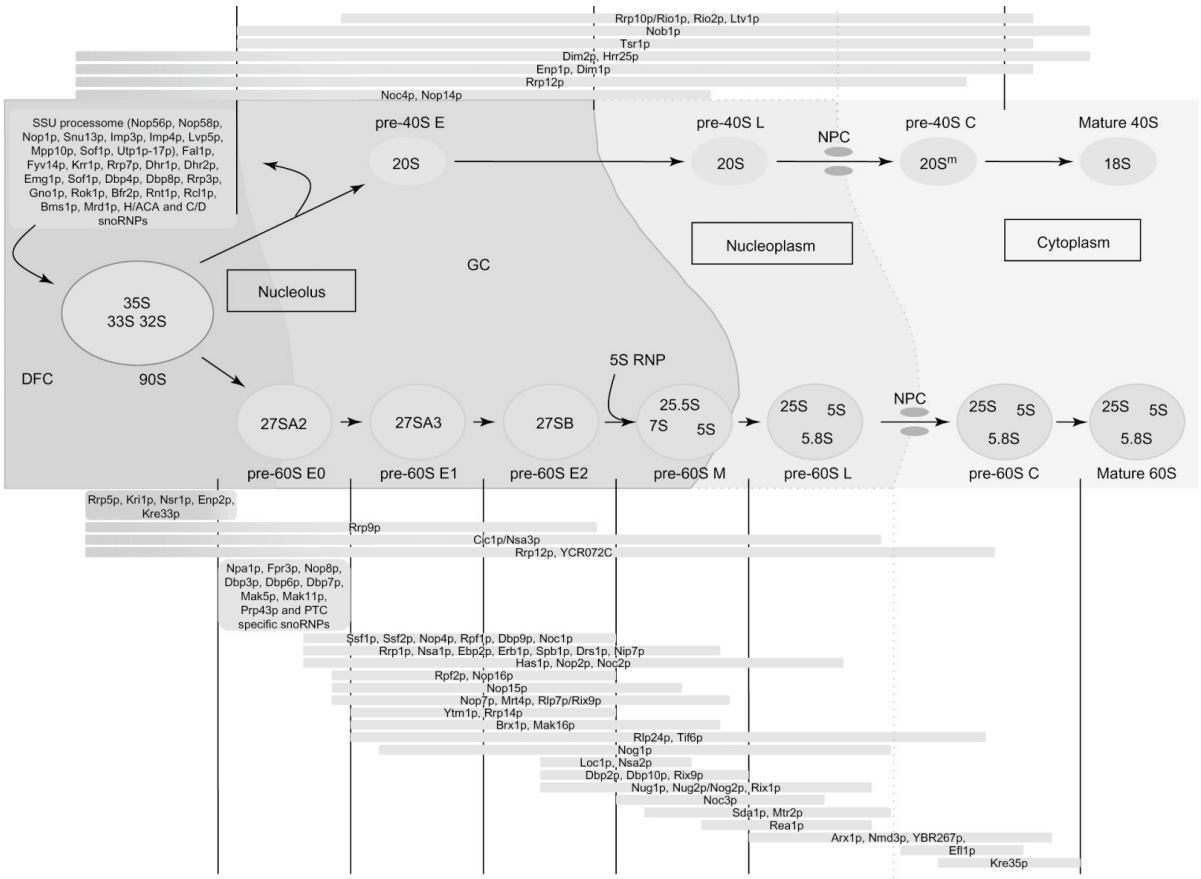


Figure 13: Assembly and disassembly of ribosome synthesis factors with the pre-ribosomal particles. Reproduced from Dez and Tollervey, 2004.

The existence of multiple pre-ribosomes is inferred from the RNA and protein composition of purified particles. The upper panel indicates 40S subunit synthesis factors. The central panel indicates the pre-ribosomal particles and their predicted sub-cellular locations. The lower panel indicates 60S synthesis factors. During ribosome synthesis, the 35S pre-rRNA primary transcript undergoes sequential processing through the intermediates indicated (within the ovals) to the mature 18S, 5.8S and 25S rRNAs. The pre-rRNA is transcribed in the dense fibrillar component (DFC) of the nucleolus and the maturing pre-ribosomes move from the DFC to the more peripheral granular component (GC) of the nucleolus. The particles are then released into the nucleoplasm, prior to transport through the nuclear pore complex (NPC) into the cytoplasm. The initial particles, termed 90S pre-ribosomes or the small subunit (SSU) processome, largely contain factors that are required for 40S subunit synthesis. Factors required for 60S synthesis, largely associate with the pre-60S particles during their maturation following separation from the pre-40S pathway. These pre-ribosomes are classed as early (E), middle (M), late (L) and cytoplasmic (C) (indicated by the names outside the ovals). A base dimethylation modification (m) within the 20S pre-rRNA has been used as a signature for late pre-40S particles. During the course of the maturation process, a large number of ribosome synthesis factors are bound and released. For each factor, the pre ribosomal particles in which they have been identified by proteomic analyses are indicated. The 5S rRNA is independently transcribed and incorporated into the pre-60S M particle as a 5S ribonucleoprotein (5S RNP) particle.

2.3.1. An early common pre-ribosome complex: the 90S particle.

Recently, large ribonucleoprotein complexes involved in early steps of ribosome biogenesis were isolated in the Baserga and Hurt laboratories (Dragon et al., 2002; Grandi et al., 2002).
In a search for proteins associated with the U3 snoRNA, the Baserga laboratory, with the help of a known U3 associated protein as bait, isolated a particle containing 28 associated proteins. Among these 17 proteins (**U**-**t**hree associated **p**roteins or **Utp**1-17p) were uncharacterised, 10 were previously described to be Utp's, and one protein was already known but not described as a Utp. This complex was estimated to sediment around 80S and was termed **SSU processome** by analogy with the splicesome (see Fournier and Maxwell, 1993).
Most of these factors are localised in the nucleolus, and loss of function analyses demonstrate that these factors are required for early processing events at A0, A1 and A2 (Dragon et al., 2002; Bernstein et al., 2004).
In addition, after depletion of essential Utps or the U3 snoRNA, the terminal knobs observed at the 5' ends of the nascent pre-rRNA transcript after chromatin spreading (Miller and Beatty, 1969), were lost (Mougey et al., 1993; Dragon et al., 2002). It is likely that these terminal knobs correspond to the RNP complex described above suggesting a co-transcriptional association of the SSU processome with the nascent pre-rRNA.
More recently, 12 additional components associated to the SSU processome were reported from the Baserga lab (Bernstein et al., 2004).

Starting with 13 bait-proteins identified previously (Gavin et al., 2002) the Hurt laboratory characterised a particle with a sedimentation coefficient estimated to 90S (Grandi et al., 2002). According to the authors, this 90S particle contains 35 ribosome biogenesis factors, the U3 snoRNA, and the 35S pre-rRNA. Interestingly, this 90S particle is enriched in 40S biogenesis factors and lacks 60S synthesis factors, suggesting that these factors bind later or that their affinity for the 35S pre-rRNA is low.

Noteworthy, the SSU processome protein composition (Dragon et al., 2002; Bernstein et al., 2004) correlates with the 90S particle protein content, suggesting that these two particles are almost identical (see discussion in Bernstein et al., 2004; Granneman and Baserga, 2004).
However, one can still ask whether "the" 90S pre-ribosome described in the 1970s has been really isolated. In fact, most of the 90S/ SSU processome factors described can efficiently coprecipitate 35S and 23S pre-rRNA with the same efficiency, while 20S pre-rRNA is poorly

coprecipitated (see Bernstein et al., 2004; Granneman and Baserga, 2004 and Fig. 28). The assignment of the rRNA species of the particle isolated by Grandi et al (Grandi et al., 2002), is based on primer extension analysis, but it is important to note that in these experimental conditions the 35S and 23S pre-rRNAs are not discriminated. Therefore it is not clear whether these complexes described above do represent "the" 90S pre-ribosome (Granneman and Baserga, 2004). Anyway it is likely that these complexes represent an early intermediate of the LSU and SSU since the rRNA protein content differ from the following 66S and 43S precursors.

As described earlier (see introduction 2.2.2.3.), it was suggested that the U3 snoRNA triggers a major rRNA structural reorganisation to facilitate rRNA maturation and r-protein (?) assembly. Therefore, one can speculate that the SSU processome components could serve as a platform to maintain a favourable structural organisation of the rRNA facilitating on one side proper rRNA folding, maturation and r-proteins assembly, and on the other side avoiding formation of inhibitory rRNA folding and r-protein assembly.

2.3.2. Nuclear/ nucleolar assembly events of LSU and SSU precursors.

2.3.2.1. Non-ribosomal constituents of pre-60S particles.

As indicated earlier, maturation of LSU rRNA after cleavage at A2 or A3 proceeds through different rRNA intermediates, thus suggesting that probably several 66S pre-particles can be isolated and distinguished according to their rRNA/ ribosome biogenesis factor content and cellular localisation.

Indeed, recent work reveals that the 66S particle is heterogeneous and highly dynamic. Several pre-66S intermediates were isolated and were classified according to their ribosome biogenesis factor/ RNA composition and cellular localisation as **pre-66S e**arly, **m**iddle and **l**ate (**pre-66S-E**, **pre-66S-M**, **pre-66-L**, respectively) and additional sub-complexes (Fig. 12-13). Probably, further analysis will reveal subtle protein composition differences which will lead to the identification of even more intermediates (reviewed in Fromont-Racine et al., 2003).

Overall, these studies showed that over 70 proteins are associated and required at different steps of the LSU rRNA maturation pathway. Interestingly, the composition of the 66S particles is highly dynamic since many of the factors associate and dissociate from the particles (see Fig. 13 and Milkereit et al., 2001). Among these factors several are predicted to be nucleotide binding proteins, like GTPases and AAA-type ATPases which could be involved in structural rearrangement, or dissociation/ association events, or serve as molecular motor for intra-nuclear trafficking. In fact, recent work gave evidence that the ATPase activity of a ribosome biogenesis factor triggers a structural rearrangement of the LSU precursor (Nissan et al., 2004).

Finally, around 50 non-ribosomal proteins are associated with the earliest nucleolar pre-60S ribosomes, but only 5 factors are present on the most mature pre-60S subunits after export to the cytoplasm (see Fig. 13 and Nissan et al., 2002).

2.3.2.2. Non-ribosomal constituents of pre-40S particle.

In contrast to the pre-66S particle(s), the composition of the 43S particle appears to be less complex. Apparently, cleavages at A0, A1 and A2 are accompanied with the release of almost all the ribosome biogenesis factors contained in the SSU processome. Strikingly, the nucle(ol)ar 43S particle carries only eight major non-ribosomal proteins. Most of these remain associated during export to the cytoplasm, where they are likely to participate in the last steps of SSU maturation.

Conditional mutants analyses suggest that many of these factors are required for efficient pre-40S nuclear export and/ or late cytoplasmic maturation events.

Furthermore, the 43S particles, in contrast to the 66S particles, do not contain any GTPase, or ATPase, but at least 3 of the 43S associated ribosome biogenesis factors were described to possess kinase activity (Vanrobays et al., 2001; Schafer et al., 2003; Vanrobays et al., 2003; LaRonde-LeBlanc and Wlodawer, 2005; Schafer et al., 2006).

2.3.3. Nucleo(lar)-cytoplasmic transport events in eukaryotic ribosome biogenesis.

Beyond, rRNA transcription, maturation, modification, and assembly intense intra-cellular trafficking in eukaryotes is required to ensure proper delivery and formation of translational active ribosomes to the cytoplasm.

In *S. cerevisiae*, in optimal growth conditions around **4000 ribosomal subunits per minute are exported** from the nucleus to the cytoplasm. This implies that at least as many of the 78□–proteins have to be imported into the nucle(ol)us, in addition to the transport of the r–protein mRNA´s and the numerous ribosome biogenesis factors.

2.3.3.1. The import/ export machinery: general facts and principles.

The passage of most of the substrates through the nuclear envelope requires a transport machinery, which is constituted of the **N**uclear **P**ore **C**omplex (**NPC**) embedded in the nuclear envelope, and **soluble factors** able to recognise and direct the substrate through the pore.

Bi-directional exchange across the nuclear envelope occurs via the NPC a huge macromolecular protein complex (50-60 MDa in yeast).
The NPC allows and controls exchange of molecules between the nucleus and the cytoplasm. While ions, small metabolites and proteins under 40 kDa can freely diffuse (Gorlich and Kutay, 1999; Wang and Brattain, 2007), most larger molecules or complexes movement across the NPC diffusion barrier is mediated by specific transport receptors (Rodriguez et al., 2004; Pemberton and Paschal, 2005; Kohler and Hurt, 2007).

Protein composition of the NPC was largely studied in many eukaryotes by biochemichal and/ or genetic analysis. Despite size variation of the NPC between different organisms analysed, these studies revealed a remarkable structure homology. The NPC consists of three major domains, the **central core**, **nuclear basket**, **cytoplasmic fibrils** which in yeast are formed by the repetitive assembly of around 30 proteins: the **nucleoporines** (see Fig. 14A). The NPC can dilate in response to the translocation of large cargoes, up to 40 nm (Kiseleva et al., 1998). Among these nucleoporines, eleven are characterised by the presence of phenylalanine-glycine repeats (**FG repeats**) separated by hydrophilic linkers. Apparently, the FG-rich repeats are essential for cell viability although their amount can be reduced (see Strawn et al., 2004; Chandler, 2005). Facilitated passage through the NPC requires interactions of the karyopherin with the FG-repeats (Iovine et al., 1995; Radu et al., 1995; Bayliss et al., 1999; Bayliss et al., 2000; Bednenko et al., 2003). Furthermore, recent work indicates that the FG repeats form an elastic reversible hydrogel mesh-like structure, probably responsible for NPC selectivity. In this condition, the transport receptors overcome the selectivity barrier by

catalysing their own nuclear pore-passage by a competitive disruption of adjacent inter-repeat contacts, which transiently opens adjoining meshes (Frey et al., 2006).

Interestingly, in contrast to the other nucleoporines, five of the FG-nucleoporines are asymmetrically located, however this organisation is not required to direct translocation (Zeitler and Weis, 2004).

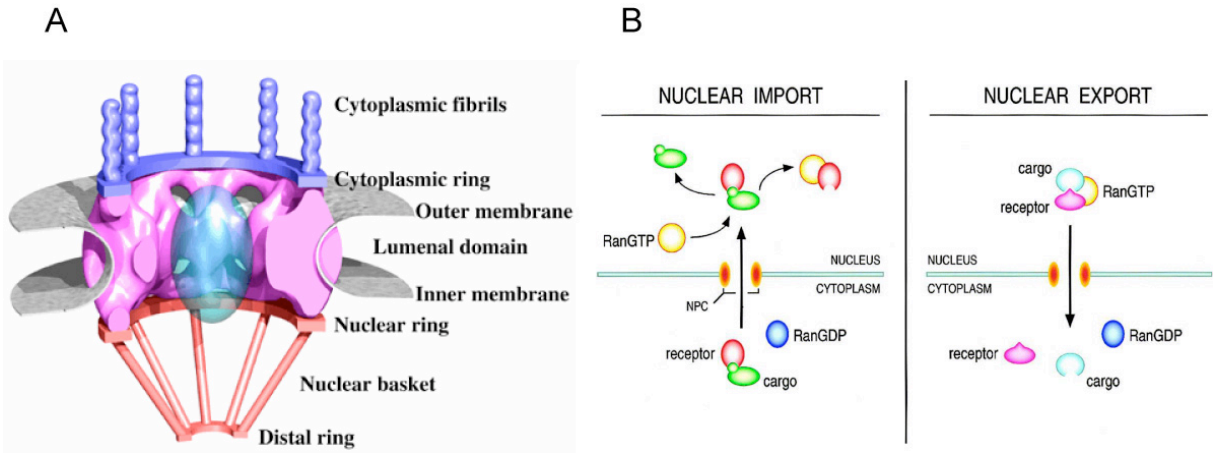


Figure 14: The nuclear pore complex and the ran cycle.

(A) Nuclear pore complex (NPC). A 3D reconstruction of detergent extracted and negatively stained NPC is shown (Hinshaw et al., 1992. see also introduction 2.3.3.1. for details).

(B) The differential effects of RanGTP on nuclear import and nuclear export receptor-cargo complexes. Import receptors bind their cargos in a RanGTP-independent manner and RanGTP causes dissociation of these complexes. They are thus permitted to form in the cytoplasm and dissociate in the RanGTP-rich nucleus. Export receptors form stable complexes with their cargos only in the presence of RanGTP. These ternary complexes are thought to be the export unit, and dissociate in the cytoplasm and/ or on the cytoplasmic filaments of the pore where RanGAP activity converts the RanGTP to RanGDP.

Active translocation of most macromolecular complexes through the NPC involves specific signals recognised by specific receptors. The substrates or cargoes contain targeting motifs (like NES or NLS) that correspond either to particular amino acids or nucleotide sequences, or conformation. With the exception of mRNA, most targeted molecules are recognised by the **karyopherin- receptor family** (also named importins and exportins) (see Gorlich and Kutay, 1999; Rodriguez et al., 2004). The importins and exportins interact with the **small GTPase Ran** (Moore and Blobel, 1993). With the help of regulatory proteins - cytoplasmic Ran GTPase activating protein (RanGAP) and the nuclear Ran guanine nucleotide exchange factor (RanGEF), a **RanGDP/ RanGTP gradient** is formed and probably directs translocation (Rodriguez et al., 2004).

The importin-cargo complex upon arriving on the nucleoplasmic side of the NPC is dissociated by the binding of RanGTP to the importin. In contrast, RanGTP binding with exportin stimulates assembly with the cargo, after transclocation through the NPC dissociation of the cargo depends on the RanGTP hydrolysis into RanGDP (see Fig. 14B).
It should be noted, that receptor-cargo complexes translocation through the NPC is independent of the energy provided by NTP hydrolysis, in fact this energy is required for receptor-cargo dissociation, thus for karyopherins recycling.

2.3.3.2. Factors involved in pre-ribosome nucleo(lar)-cytoplasmic export.

Most of the r-proteins are predicted to contain a NLS, however, because of their small size most of them could be imported by simple diffusion process. However, it seems that efficient r-protein import is mediated by several r-proteins "specific" importins (Rout et al., 1997; Schlenstedt et al., 1997; Pemberton and Paschal, 2005).

Ribosomal subunit nuclear export is still poorly understood. Microinjection experiments in *X. laevis* oocytes have shown that **ribosome export is energy and temperature dependent and saturable**. In order to understand whether the ribosome components contain essential information(s) required for nuclear export, microinjection experiments were performed using ribosomes isolated from prokaryotic and eukaryotic cells. The authors of these two independent studies conclude that eukaryotic ribosomes contain conserved information(s) sufficient to stimulate ribosome nuclear export, while conflicting results regarding the export competence of prokaryotic ribosomes are reported (Khanna-Gupta and Ware, 1989; Bataille et al., 1990). Of course, it is somehow provocative to think that prokaryotic ribosomes are competent for nuclear export, this would suggest that the export machinery recognises conserved ribosome constituents and/ or structural landmark(s). At least this appears to be true for eukaryotic ribosomes. In addition, ribosomal subunit export is directional and selective. In yeast, only pre-40S particles containing 20S pre-rRNA and pre-60S particles containing 25S, 5.8S and 5S rRNAs are *bona fide* substrates for the export machinery (Trapman and Planta, 1976; Trapman et al., 1976).

More recently, GFP tagged r-protein reporters and/ or *in situ* localisation of pre-rRNA were used to screen mutants and/ or inhibitors affecting ribosomal subunits export (Hurt et al., 1999; Moy and Silver, 1999; Stage-Zimmermann et al., 2000; Gadal et al., 2001; Milkereit et

al., 2001; Grandi et al., 2002). In summary, it was shown in all eukaryotic organisms studied so far, that both ribosomal subunits export depends on the GTP/ GDP binding protein Ran (see Fig. 15).

Furthermore, several nucleoporines were described to be involved in nuclear export of pre–ribosomes, but since many of these participate in the traffic of other substrates it is difficult to determine whether they play a direct or indirect role in pre-ribosome export. However, in some cases some nucleoporines mutations were shown to only affect pre–ribosomal subunit export indicating a specialised molecular function of the NPC components in this process (Gleizes et al., 2001).

In addition, it was shown that the exportin **Crm1p/ Xpo1p** plays a crucial role in the export of both ribosomal subunits. The exportin Crm1p/ Xpo1p binds proteins containing NES in a RanGTP dependent manner (Thomas and Kutay, 2003), suggesting that either this exportin binds directly to r-protein(s) and/ or to protein adapter(s) containing an NES sequence and being able to bind to ribosome structural component(s) (see Fig. 15).

Such a protein adapter, Nmd3p, was described to be required for pre-60S nuclear export, and to be associated with the LSU precursor (see Fig. 15 Ho and Johnson, 1999; Ho et al., 2000; Gadal et al., 2001; Johnson et al., 2002; Trotta et al., 2003). Genetic and biochemical analyses were first suggesting that Nmd3p is recruited to the ribosome by rpL10. However in the course of this work, recent data suggested that rpL10 incorporation into pre-ribosomes triggers the release of Nmd3p from the newly exported LSU (Hedges et al., 2005; West et al., 2005). Nmd3p binds one of the ribosome constituents, which remain to be determined. In addition, other ribosome biogenesis factors were suggested to participate in ribosomal subunit export, but their exact function in this step remain to be clarified (for review see Johnson et al., 2002; Fromont-Racine et al., 2003).

Finally, recently two other transport receptors were suggested to be involved in LSU export (see Fig. 15 and Bradatsch et al., 2007; Yao et al., 2007)

Although some ribosome biogenesis factors were suggested to be required for SSU export (Milkereit et al., 2003b; Schafer et al., 2003; Seiser et al., 2006 among others) such a protein adapter bridging the export machinery to the export competent SSU still has to be identified.

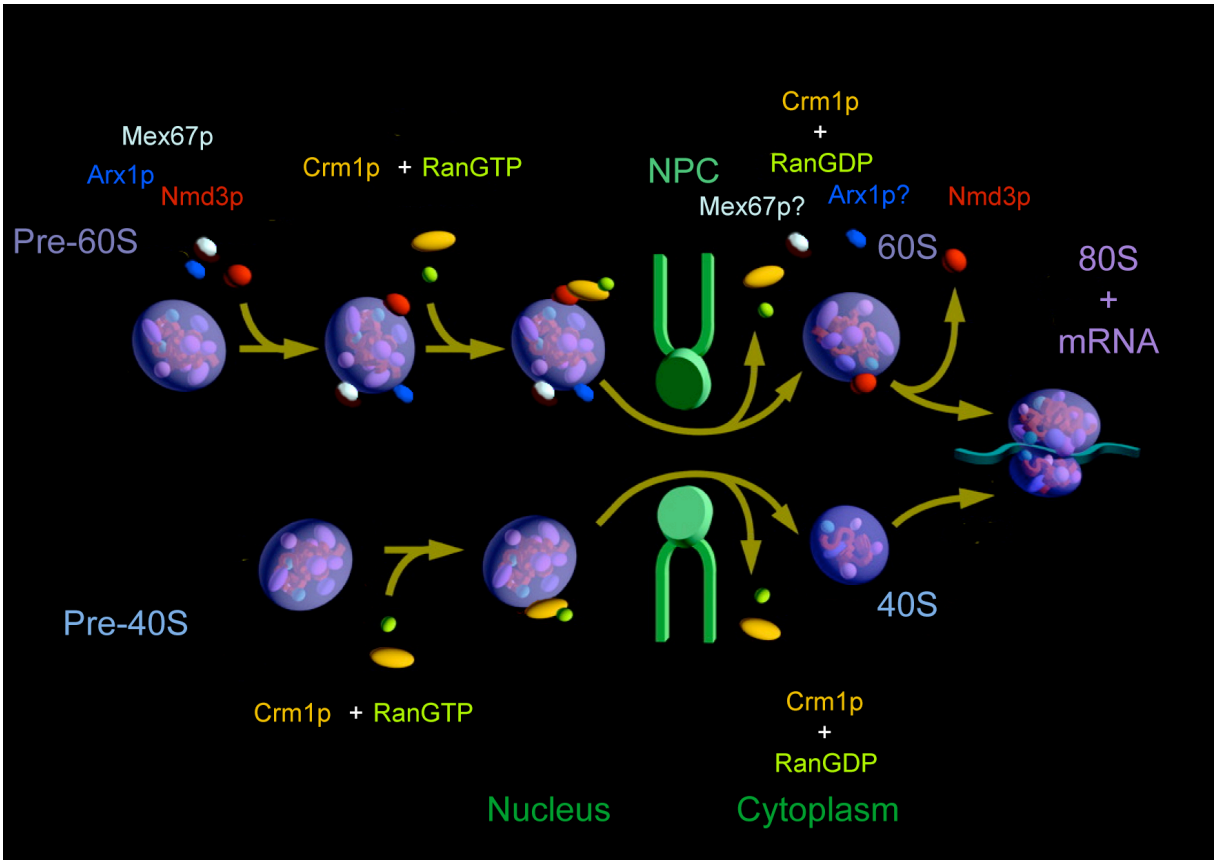


Figure 15: Current model of pre-ribosomal subunit export.
Modified from Aitchison and Rout, 2000. See introduction 2.3.3.2. for details.

Interestingly, *in silico* analysis revealed that some of the ribosome biogenesis factors suggested to be involved in ribosomal subunit export, contain a structural motif, the HEAT repeat, which is one of the characteristics of the transport receptor from the karyopherin-β family (see Dlakic and Tollervey, 2004; Oeffinger et al., 2004). The contribution of this structural motif in ribosomal subunit export remains to be determined. However it was hypothesised that these factors would participate in ribosomal subunit export by shielding the negative charge of the particle thus facilitating passage through the NPC (Dlakic and Tollervey, 2004; Oeffinger et al., 2004).

2.3.4. Late cytoplasmic maturation steps of pre-ribosomes.

Exported immature ribosomal subunits are converted into mature ribosomal subunits in the cytoplasm.

The LSU is exported as 60S particle containing the mature rRNA species. Therefore it is unclear whether the large subunit is further matured in the cytoplasm. In fact, early study indicated that the newly synthesised 60S subunits were not directly incorporated into translating ribosome (Trapman and Planta, 1976). This lag in translation activation could be interpreted as being probably related in some extent to cytoplasmic assembly events.
Recently it was shown that the export machinery and a few ribosome biogenesis factors accompanying the LSU from the nucleus into the cytoplasm are released (and recycled) prior to engage the LSU into a translating ribosome (Saveanu et al., 2001; Senger et al., 2001; Nissan et al., 2002; Fromont-Racine et al., 2003; Lebreton et al., 2006).
Recent work suggested that the remaining factors associated with the late cytoplasmic 60S precursors are released through the action of GTPases (Saveanu et al., 2001; Senger et al., 2001; Nissan et al., 2002; Fromont-Racine et al., 2003; Kallstrom et al., 2003; Lebreton et al., 2006). Whether, these GTP-hydrolysis reactions mediate other structural rearrangements is so far unknown.

Final cytoplasmic maturation of the 43S precursor was described already more than 30 years ago (Udem and Warner, 1973), but it is only recently that several factors involved in this late maturation step were described (for review see Fromont-Racine et al., 2003). Among these factors, a protein family was identified by synthetic lethality screen and homology searches (Vanrobays et al., 2001; Geerlings et al., 2003; Schafer et al., 2003; Vanrobays et al., 2003; Schafer et al., 2006). Interestingly, these factors - Rio1p and Rio2p - are members of a serine kinase family. In the course of this work it was shown that Hrr25p dependent phosphorylation followed by dephosphorylation trigger SSU structural rearrangement(s) (Schafer et al., 2006).
In addition it was suggested that, the protein dependent base dimethylation near the 3’ end of the 18S rRNA occurs in the cytoplasm (Lafontaine et al., 1994; Lafontaine et al., 1995; Lafontaine et al., 1998; Fromont-Racine et al., 2003; Vanrobays et al., 2004).

3. Functions of ribosomal proteins.

3.1. Contribution of r-proteins to ribosome biogenesis.

3.1.1. The eubacterial r-protein assembly: a paradigm and a paradox.

Most of our knowledge about r-protein assembly comes from the pioneering work that started in the mid- 1960s on eubacterial ribosomes (see below for details).

The main conclusions of these studies can be summarised as the following: (1) both prokaryotic **ribosomal subunits can be assembled *in vitro* from their purified components** (rRNA and r-proteins); (2) r-proteins assemble according to a **hierarchical order**; (3) *in vitro* reconstituted ribosomal subunits are competent in translation. These experiments indicate that the eubacterial ribosome is a **self-assembling macromolecular machinery**, in other words: the total information for the assembly pathway as well as the quaternary structure of the active ribosomes resides completely in the r-proteins and rRNA primary sequences. However, with the development of eubacterial genetic analysis, and *in vivo* analysis, several reports are challenging the view of how this machinery is assembled *in vivo* in prokaryotes (see below).

3.1.1.1. Conditions required for efficient *in vitro* reconstitution experiments.

Early experiments showed that r-proteins can be dissociated and isolated from mature ribosomes using high ionic strength conditions (like CsCl, LiCl). In fact several reports indicated that reconstitution of functionally active ribosomal particles from inactive partially split components was possible (see Gavrilova et al., 1966; Hosokawa et al., 1966; Lerman et al., 1966; Spirin and Belitsina, 1966; Spirin et al., 1966; Staehelin and Meselson, 1966). These experiments have opened the way to initially study the relationship between the structure and the function of the ribosomal components in protein synthesis, but also paved the way to study in more detail ribosome assembly *in vitro*. Furthermore, the use of different ionic strength and/ or denaturing conditions (e.g. LiCl, CsCl/ Urea) allowed the possibility of stepwise or complete disassembly of r-proteins and further reassembly including or omitting different r-protein fractions, followed by functional characterisation of the translation

competence in *in vitro* assays (reviewed in Spirin, 2000, see also Traub et al., 1967; Nomura and Traub, 1968; Traub and Nomura, 1968b; Traub et al., 1968).
Finally an experimental "tour de force" by Nomura and colleagues, has demonstrated that the reconstitution of both ribosomal subunits can be achieved from isolated rRNA and the complete set of purified r-proteins (see Traub and Nomura, 1968a; Nomura and Erdmann, 1970). Interestingly, for the 30S ribosomal subunit, hybrid ribosomal subunits have been reconstituted from components of different bacterial origin (Nomura et al., 1968), however *in vitro* reconstitution experiments failed when using rRNA from eukaryotic organisms (Traub and Nomura, 1968a see also Nomura, 1970).

Noteworthy, ribosome self-assembly from purified structural components is a characteristic which seems to be shared by prokaryotic ribosomes since it was demonstrated that both archaeal ribosomal subunits can be assembled *in vitro* (Londei et al., 1986; Sanchez et al., 1990; Sanchez et al., 1996).

The *in vitro* 30S ribosomal subunit reconstitution requires defined conditions. (1) A moderate ionic strength (0.3-0.5 M KCl optimum at 0.37 M); (2) a rather high Mg^{2+} concentration (10-30 mM); (3) a controlled pH (pH 6.5-8.0 at 40°C) and finally (4) an increased temperature (optimum at 40°C, the temperature dependency follows an exponential reaction with a plateau reached at 40°C)(Traub and Nomura, 1969).
Apparently, a too high ionic strength suppresses interaction between the r-proteins and rRNA, while a too low ionic strength increases non-specific interactions markedly. The relative high amount of Mg^{2+} seems to be necessary primarily for the maintenance of the rRNA tertiary and secondary structure, thus providing a scaffold for r-protein arrangement. Finally, an elevated temperature is believed to be necessary to facilitate the structural rearrangements of an intermediate RNP from a less compact to a more compact conformation (see Spirin, 2000; Nierhaus, 2004 and below).
The total *in vitro* reaction is a one step procedure and can be summarised as following (1):

$$16S\ rRNA + TP30 \xrightarrow{[20\ mM\ Mg^{2+},\ 40°C,\ 20\ min]} 30S \quad (1).$$

Where TP30 is: total r-proteins derived from 30S subunits. It should be noted that usually TP30 is present in several molars excess in comparison to the rRNA (Maki and Culver, 2005).

In fact, the rate-limiting step of *in vitro* 30S assembly is apparently the relative strong temperature dependency, which is required for the "activation" of an assembly intermediate as described in equation (2).

$$RI_{30} \xrightarrow{[20\ mM\ Mg^{2+},\ 40°C,\ 20\ min]} RI^*_{30} \quad (2).$$

First, a low temperature (0°C ≤ T° ≤ 20°C) is sufficient to form the **RI_{30}** intermediate, containing (S4-S9, S11-S13, and S15-S20). Second the temperature step leads to the formation of the **RI^*_{30}** without change in term of r-protein composition. Finally, only the RI^*_{30} can assemble the remaining r-proteins (S2, S3, S10, S14, and S21) at any temperature (Traub and Nomura, 1968a; Traub and Nomura, 1969; Maki and Culver, 2005).

The reconstitution of the 50S ribosomal subunit as described by Nierhaus and colleagues (Nierhaus and Dohme, 1974) requires defined conditions too, which are relatively similar to the 30S assembly conditions, except that for the efficient reconstitution a two steps procedure is applied. (1) A moderate ionic strength (400 mM NH_4Cl) (2) a rather high Mg^{2+} concentration in the second step (20 mM); and finally (3) two different temperature steps are required for efficient reconstitution (Nierhaus and Dohme, 1974 and reviewed in Nierhaus, 2004).

The 50S *in vitro* reconstitution procedure can be summarised as following (1) and (2):

$$(23S+5S)\ rRNA + TP50 \xrightarrow{[4\ mM\ Mg^{2+},\ 44°C,\ 20\ min]\quad[20\ mM\ Mg^{2+},\ 50°C,\ 90\ min]} 50S \quad (1).$$

Where TP50 is: total proteins derived from 50S subunits.

Apparently the two-steps procedure is a consequence of the fact that the rate limiting steps of early and late assembly involve conformational changes that differ in their ionic optima *in vitro* [see Nierhaus and Dohme, 1974 and equation (2)].

$$RI_{50}(1) \xrightarrow{T_1°C} RI^*_{50}(1) \xrightarrow{T_2°C} RI_{50}(2) \longrightarrow 50S \quad (2).$$

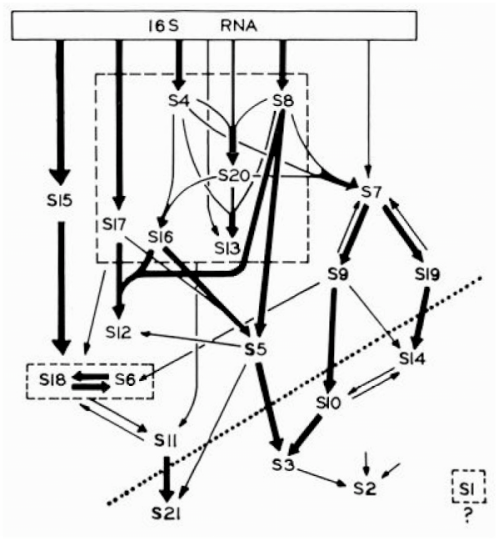
Accordingly, three reconstitution intermediates have been characterised [see equation (2)]. The **RI_{50} (1)** and **RI^*_{50} (1)** intermediates are identical in their rRNA/ r-proteins composition (around two-thirds of the LSU r-proteins) despite the drastic difference in their respective sedimentation value (33S and 41S respectively). In contrast, the third intermediate **RI_{50} (2)** contain all the components of the active 50S subunit but is inactive in translation (Nierhaus and Dohme, 1974; Nierhaus, 2004). Thus, it appears that here also the rate-limiting step of active 50S subunit reconstitution is the temperature dependent conformational change.

Reconstitution of the bacterial ribosomal subunits are very efficient and reach usually 50%-100% of the input material (see Nierhaus, 1991).
However, the reconstitution conditions are clearly non-physiological, suggesting that although self-assembly can occur *in vitro*, it is evident that *in vivo* a facilitated system might have evolved to fulfil the required ribosome production at distinct growth conditions.

3.1.1.2. Assembly dependencies and co-dependencies: "*in vitro* assembly maps".

With the help of chromatography fractionation, individual r-proteins were purified in the Nomura and Nierhaus laboratories. Taking advantage of the *in vitro* system described above (see introduction 3.1.1.1.), the capacity of individual purified r-proteins to bind rRNA after combinatorial and sequential r-protein addition was analysed. The experimental results of such binding analyses are summarised in **"assembly maps"** (see Mizushima and Nomura, 1970; Held et al., 1973; Rohl and Nierhaus, 1982; Herold and Nierhaus, 1987; Nierhaus, 2004 and see Fig. 16).
According to their behaviour during these *in vitro* reconstitution experiments r-proteins were divided into three categories. One r-proteins group is designated **primary binding proteins**, since they can form stable complex with the rRNA individually and independently. Accordingly, the **secondary binding proteins** require the presence of one or several primary binding proteins to be incorporated into ribosomal subunits. Finally, the **tertiary binding protein** group requires both primary and secondary binding proteins to establish contacts with rRNA (review in Nierhaus, 2004 and see Fig. 16).

A



B

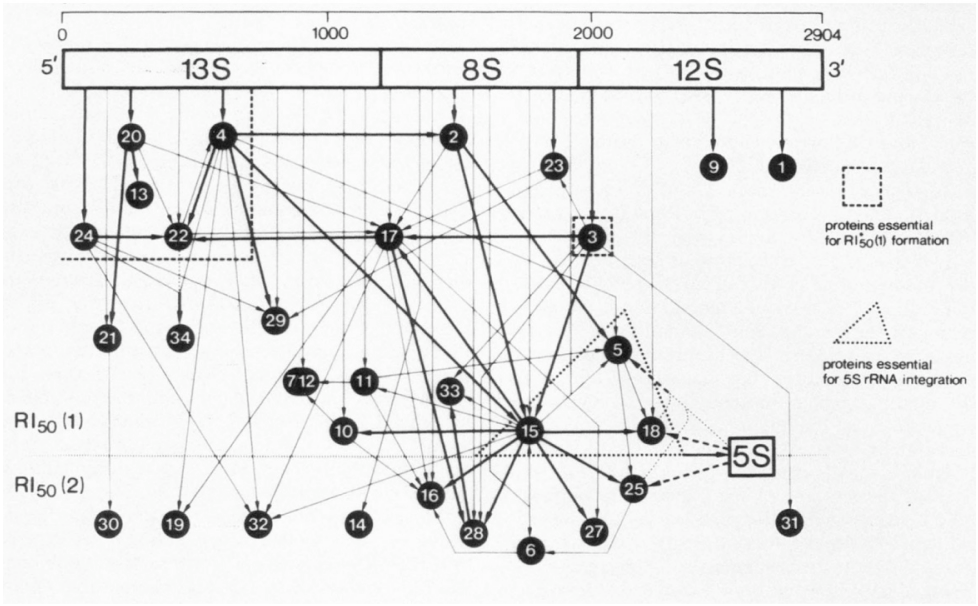


Figure 16: Ribosome *in vitro* assembly maps in *E. coli*.

(A) Assembly map of the 30S subunit. Revised assembly map of *E. coli* 30S r-proteins. Reproduced from Held et al., 1974. Arrows between proteins indicate the facilitating effect on binding of one protein on another; a thick arrow indicates a major facilitating effect. The map may be used to indicate the following relationships.

The thick arrow from 16S rRNA to S4 indicates that S4 binds directly to 16S rRNA in the absence of other proteins. The thin arrow from 16S rRNA to S7 indicates that S7 binds weakly to 16S rRNA in the absence of other ribosomal proteins. Thin arrows pointing toward S7 from S4, S8, S20, S9, and S19 indicate that the latter proteins all enhance the binding of S7 to rRNA. The arrow to S11 from the large box with dashed outline indicates that S11 binding depends on some of the proteins enclosed in the box; it is not known exactly which proteins. Proteins above the dotted line are those either required for the formation of RI* particles or found in the isolated 21S RI particles.

(B) Assembly map of the 50S subunit. Revised 50S assembly map reproduced from Herold and Nierhaus, 1987. The main fragments of 23S rRNA (13, 8, and 12S) are indicated. The proteins are roughly arranged according to their binding regions on 23S rRNA.

RNA → Lx, Lx is an rRNA binding protein; →(→), binding of Lx is strongly (weakly) dependent on Ly. The encircled proteins ••••• are essential for mediating the binding of 5S rRNA to 23S rRNA. Proteins enclosed by - - - are important or essential for the conformational change $RI^{*}_{50}(l)$ $RI_{50}(l)$. Components below . . . . are not present on the $RI_{50}(l)$ particle.

In both cases, a relatively high proportion of r-proteins can assemble with rRNA at low temperature to form the RI_{30} and RI_{50} (1) intermediates. In order, to determine whether all these r-proteins are required and sufficient for the critical transition to $RI^*_{30\ \&\ 50}$ omission experiments where performed. Surprisingly, only a few r-proteins were shown to be required and sufficient for the early assembly step (LSU: 5 required + 1 stimulating, and SSU: 5 required). Moreover, not all the primary binding proteins are required for this transition step (review in Nierhaus, 2004).

Remarkably, these early assembling r-proteins - more strikingly for the LSU - have binding sites located towards the 5' end of the rRNA. Although this repartition is not obvious for the early assembling SSU r-proteins, further experiments are indicating a 5'→ 3' directed assembly (compare Fig. 17A and 17B, see also Powers et al., 1993; Culver, 2003). In fact, the dynamic SSU r-proteins assembly was followed by chemical foot-printing analyses at different time points during the *in vitro* assembly process. These results strongly suggest a 5'→ 3' polarity during *in vitro* SSU assembly. In addition SSU r-proteins were divided in 4 groups (Early, Mid Mid-late, and Late) according to their assembly kinetics (Powers et al., 1993; Culver, 2003).

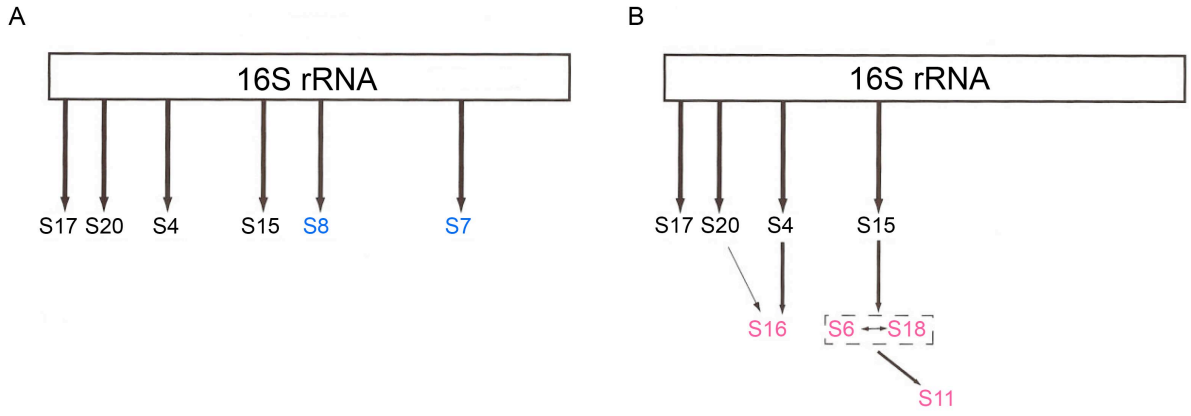


Figure 17: Schematic representation of the 5´→ 3´polarity of 30S subunit assembly. Reproduced from Culver, 2003.

(A) SSU primary binder r-proteins as determined by the assembly map (see Fig. 16).
The interaction of the six primary binding proteins with their respective 16S rRNA domains is shown. The proteins are colored to represent their groupings according to the assembly kinetic data from Powers et al., 1993; early binding proteins are shown in black and mid binding proteins are shown in blue.
(B) Dynamics SSU r-proteins assembly *in vitro*.
The interaction of early binding proteins with their respective 16 rRNA domain as described by Powers et al., 1993 is shown. The proteins are colored to represent their grouping according to the assembly map; primary binding proteins are colored black and secondary binding proteins are colored pink (see Fig. 16).

From these observations, and since RNA polymerase-dependent nucleic acids polymerisation start at the 5' end, it was hypothesised: that coupling of rRNA synthesis and ribosomal assembly would define a 5' 3' **"assembly gradient",** which states that progress of rRNA synthesis dictates the assembly progress (Nierhaus, 1980a; Nierhaus, 2004).

Interestingly structural and co-dependency assembly analyses were strongly suggesting that the SSU can be divided into independent structural and assembly domains (see Fig. 18). Indeed, it was shown using *in vitro* transcribed rRNA domains and purified ribosomal proteins, that these domains can assemble independently from each others and that the morphology of these reconstituted domains analysed by negative staining electron microscopy resemble their counterparts in the complete subunit (Samaha et al., 1994; Agalarov et al., 1998; Agalarov et al., 1999).

Such independent domains assembly was not carried out for the 50S subunit since as indicated earlier, the different domains organised in the final 3D structure produce a compact, interconnected monolithic mass (see introduction 1.2.2.).

3.1.1.3. Ribosomal RNA structural rearrangements in the course of r–protein assembly.

It is clear from the ribosomal subunit 3D structure that proper ribosome assembly requires highly dynamic conformational changes of both rRNA and r-proteins.
The successful *in vitro* reconstitution of such RNP complexes - the eubacterial ribosomes - from its components provides a useful system, to elucidate the molecular mechanism of RNA-protein and protein-protein recognition, and to understand how these interactions influence protein and rRNA folding.

Despite many efforts, the process by which rRNA and r-proteins fold into functional three-dimensional structures is still poorly understood.

A variety of techniques have been used to study r-protein-rRNA interactions and RNA conformational changes.
Early studies used RNase cleavage techniques to identify elements of rRNA that interact with r-proteins (see for examples Muto et al., 1974; Zimmermann et al., 1974).

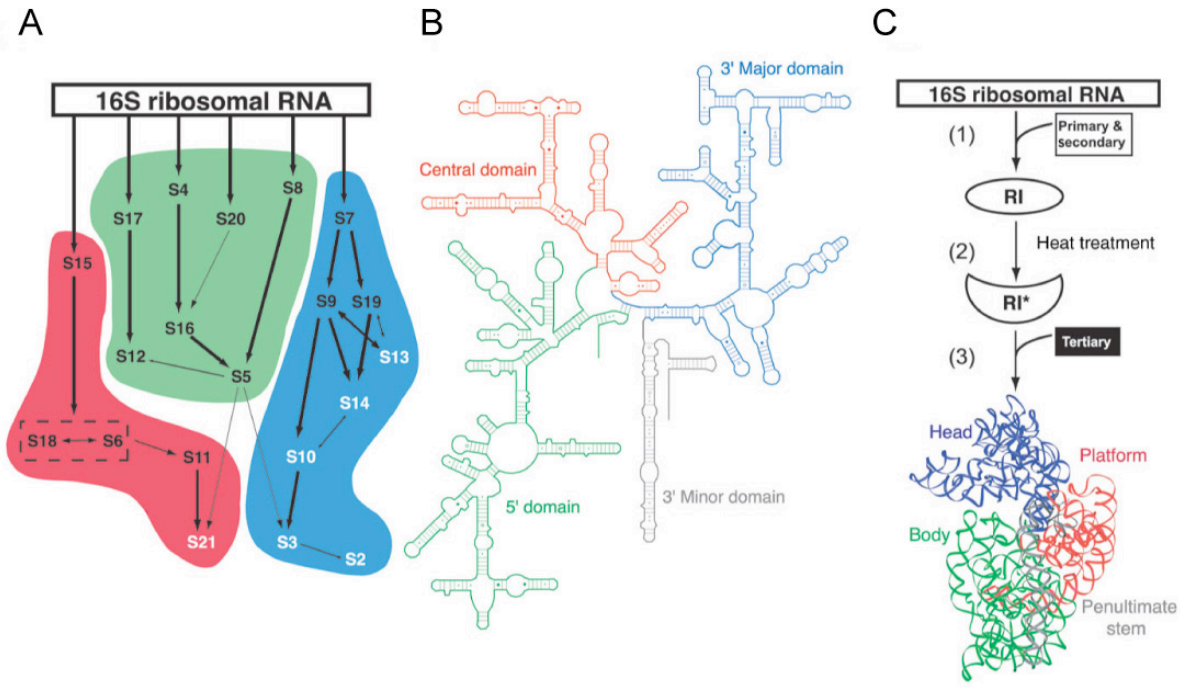


Figure 18: Three-dimensional SSU domain assembly. Reproduced from Holmes and Culver, 2004.
(A) *In vitro* 30S subunit assembly map. 16S rRNA is represented as a rectangle and arrows indicate dependency between components for association. Primary and secondary binders r-proteins are black and tertiary binders r-proteins are white. S6 and S18 are enclosed in a box to indicate that they bind as a heterodimer. The areas shaded red, green and blue indicate those proteins found in the platform, body and head of the 30S subunit, respectively.
(B) Schematic representation of the secondary structure of 16S rRNA. The four major domains of 16S rRNA, the 5', central, 3' major and 3' minor domains, are green, red, blue and gray, respectively. This color scheme is coordinated with shaded areas in a way that 16S rRNA and associated proteins are similarly colored.
(C) Schematic representation of *in vitro* 30S subunit assembly. 16S rRNA is represented by a rectangle, the reconstitution intermediate (RI) is represented by an ellipse, the 'activated intermediate' (RI*) is represented as a crescent shape and 16S rRNA from the 30S structure of *T. thermophilus* (Wimberly et al., 2000) is color-coded similarly to panel (B). (1) 16S rRNA combines with a subset of r-proteins (S4-S9, S11-S13 and S15-S20) at low temperatures to form a stalled intermediate, RI. (2) RI is converted to the activated intermediate RI* by heat treatment. (3) The remaining r-proteins (S2, S3, S10, S14 and S21) bind to RI*, forming 30S subunits.

The opposite approach - proteolytic digestion of r-proteins - was used to identify specific amino acids in these proteins that function in rRNA binding and ribosome assembly (see among others Changchien and Craven, 1978; Changchien and Craven, 1979).

Biophysical methods like difference circular dichroism and chemical iodination were employed to study global protein conformational changes in the course of ribosome assembly. In a similar way rRNA conformational changes were studied by difference ultraviolet absorption spectroscopy and difference circular dichroism (see among others Dunn and Wong, 1979c; Dunn and Wong, 1979b; Dunn and Wong, 1979a). All together these

experiments suggest that conformational changes of both rRNA and r-proteins occur in the course of ribosome assembly.
These observations are clearly supported by high-resolution scanning transmission electron microscopy analysis in the course of *in vitro* 30S assembly (Mandiyan et al., 1989; Mandiyan et al., 1991). These analyses elegantly show how the ribosomal subunit assembly is accompanied by stepwise conformational changes, compaction, with the occurrence of several structure-related intermediates.

Remarkably, RNA footprinting experiments took advantage of information collected from the *in vitro* ribosomal subunit assembly maps to identify sites of interaction between rRNA and each r-proteins (see among others Stern et al., 1989; Powers and Noller, 1995a)
In addition to direct protection effects, it was proposed that some proteins might act cooperatively to stabilise a conformational change within 16S rRNA. Some of the protection pattern observed in these footprint experiments likely result from r-protein induced rRNA folding, instead of direct protein/ RNA contacts (see Stern et al., 1989). One example of such a conformational rearrangement within a 16S rRNA containing RNP complex is well documented. Base-specific chemical footprinting experiments revealed that there is a temperature-dependent conformational change within the S4 –16S rRNA complex (Powers and Noller, 1995b). Since S4 was described as being a primary binder, and suggested to be an assembly initiator *in vivo* this rearrangement may play a critical role in assembly (Nierhaus, 2004).
Studies were also designed to use the chemical probing approach to study the dynamics of subunit assembly (Powers et al., 1993; Holmes and Culver, 2004; Holmes and Culver, 2005). These studies took advantage of changes in the rate of 30S subunit assembly at different temperatures. Changes in the higher order folding and structure of 16S rRNA at different stages of reconstitution, and therefore 30S subunit assembly, could be analysed by base-specific chemical probing of 30S subunit reconstitution reactions at various times and temperatures.

While these kinetic studies sampled a more dynamic population than previous experiments many aspects of real-time rRNA folding and the roles played by the ribosomal proteins in this folding process remain elusive.

3.1.1.4. Eubacterial r-proteins assembly *in vitro* and *in vivo*: different or similar?

How ribosomal subunits assemble *in vivo* is still poorly understood.

As described earlier, *in vitro* reconstitution experiments have described several ribosomal subunit assembly intermediates (RI) (see introduction 3.1.1.1. and 3.1.1.2.).
Indeed, ribosomal subunit precursors were isolated *ex vivo* and their rRNA and r-proteins compositions were analysed.
Apparently, two different intermediate particles were described for 30S assembly, and termed $p_1$30S and $p_2$30S (p for precursor). The $p_1$30S intermediate contains immature 17S rRNA (see ealier in this introduction 2.2.2.1.) and the r-protein content is very similar to the RI_{30} r–proteins composition. The $p_2$30S contains the full complement of r-proteins like the RI^*_{30} particle, but still contains immature 17S rRNA suggesting that the final rRNA maturation steps probably occur after r-protein assembly *in vivo* (Srivastava and Schlessinger, 1990; Nierhaus, 2004).

The *in vivo* 50S assembly occurs via three precursor particles - $p_1$50S, $p_2$50S and $p_3$50S – sedimenting with 34S, 43S and near 50S respectively (Lindahl, 1975 and references therein) which are similar to the sedimentation behaviour of the corresponding *in vitro* reconstituted RI_{50} intermediates (see introduction 3.1.1.1.).
Like for the 30S precursors, the 50S precursors r-proteins composition *in vivo* is very similar to the *in vitro* assembled RI_{50} intermediates r-proteins composition (Nierhaus et al., 1973; Nierhaus, 1991).

As indicated previously, *in vitro* assembly reconstitution experiments suggest the existence of a preferred 5’ 3’ **“assembly gradient”**. In addition, early chromatin spreads analysed by electron microscopy suggest that ribosomal subunit assembly occurs co-transcriptionaly (Miller et al., 1970; Hofmann and Miller, 1977). Thus, it was hypothesised, that coupling of rRNA synthesis and ribosomal assembly would define a 5’ 3’ **“assembly gradient”,** which states that progress of rRNA synthesis dictates the progress of assembly (Nierhaus, 1980b; Nierhaus, 2004). Therefore, *in vivo* an assembly gradient could avoid premature inhibitory rRNA rearrangements and facilitate assembly.

The non-physiological conditions used for prokaryotic *in vitro* ribosomal subunit assembly strongly suggest that *in vivo*, non-ribosomal factors might facilitate ribosomal subunit assembly.

The description of such factors is still limited (see Maki et al., 2002; Alix and Nierhaus, 2003; Maki et al., 2003; Nierhaus, 2004; Maki and Culver, 2005). One of the best example of factor facilitated ribosomal subunit assembly was recently published (Maki et al., 2002; Alix and Nierhaus, 2003; Maki et al., 2003; Nierhaus, 2004; Maki and Culver, 2005). In order to identify such a factor, the Culver group, took advantage of the 30S ribosomal subunit *in vitro* assembly approach. They have used a whole cell extract fractionation strategy to isolate molecular components that allow reconstitution of the active 30S subunit independently of the normally required temperature activation step. They could demonstrate that addition of the DnaK chaperoning system allows to bypass the temperature dependent conformational change (Maki et al., 2002; Alix and Nierhaus, 2003; Maki et al., 2003; Maki and Culver, 2005). These results are still under debate (Alix and Nierhaus, 2003; Maki et al., 2003; Maki and Culver, 2005), however they will probably serve as a reference for future screening approaches in order to identify factors facilitating ribosomal subunit assembly.

One other way to test the accuracy of the *in vitro* assembly maps is to test the consequence of r-proteins loss of function on ribosomal subunit assembly. Unfortunately, because of the lack of an efficient genetic system these approaches are scarce in the literature. Although, the *E. coli* genome sequence was published 10 years ago (Blattner et al., 1997), it is only recently that systematic single gene knockout mutants were described (Baba et al., 2006, see also profiling of *E. coli* chromosome at www.shigen.nig.ac.jp/ecoli/pec/index.jsp).

Nevertheless, it was shown that some r-proteins were not essential for cell viability (Dabbs, 1991). At least one primary binder – L24 - required for the early RI_{50}* activation step was shown to be dispensable *in vivo* for proper 50S assembly (Herold et al., 1986; Dabbs, 1991; Nierhaus, 2004). In the course of this work, a SSU r-protein primary binder was shown to be not required for cell viability. Furthermore, analysis of r-protein composition of ribosomes isolated from this mutant revealed that the downstream dependent r-proteins were still assembled (Bubunenko et al., 2006). In addition, a systematic gene knockout analysis of SSU r-proteins has confirmed previous results (review in Dabbs, 1991; Nierhaus, 2004) and

suggests that another primary binding r-protein is not essential for cell viability (Bubunenko et al., 2007).

Out of 21 SSU r-proteins 6 were suggested to be not required for cell viability. All six non-essentials r-proteins are located along the rim of the platform side away from the functional centre of the ribosome. Half of them are considered from the *in vitro* assembly maps to be crucial for proper assembly of secondary or late assembling r-proteins (Bubunenko et al., 2007).

These results are quite unexpected, either the principles of the cooperative and ordered r-proteins assembly into ribosomes that have been deduced from *in vitro* experimentation may not quite mimic the ribosome biogenesis *in vivo*. Or alternatively, the order of protein assembly into ribosomes may be somewhat different *in vivo*, with the existence of possible (less efficient?) alternative assembly pathways to counteract consequences of r-protein loss of function.

In conclusion, while many experiments suggest a similarity between *in vitro* and *in vivo* ribosomal subunit assembly, some results suggest that *in vivo* ribosomal subunit assembly might be different or more flexible to various conditions. Further experiments will have to determine the degree of similarity and differences between the *in vivo* and *in vitro* ribosomal subunit assembly pathways in prokaryotes.

3.1.2. The eukaryotic r-protein assembly.

Despite many efforts in the field the knowledge about eukaryotic r-protein assembly both *in vivo* and *in vitro* is limited.

3.1.2.1. *In vitro* assembly experiments.

Based on the experimental conditions used for bacterial ribosomal subunit *in vitro* assembly, eukaryotic ribosomal subunits *in vitro* reconstitution experiments were carried out. However the success of these reconstitution experiments was limited and so far did not give the possibility to study *in vitro* r-proteins assembly in detail.

In fact, first partial reconstitution of both ribosomal subunits were achieved in lower and higher eukaryotes (Reboud et al., 1972; Vioque et al., 1982; Lavergne et al., 1988).
However complete *in vitro* reconstitution from purified components seems to be problematic. One report of *in vitro* reconstitution of both ribosomal subunits can be found in the literature (Mangiarotti and Chiaberge, 1997). In this study, ribosomal subunits from *Dictyostelium discoideum* were reconstituted *in vitro*. However the results indicate that unlike prokaryotes, a partially purified nuclear fraction containing RNA (most probably snoRNA) is required for *in vitro* assembly. Furthermore, *in vitro* reconstitution is more efficient when the rRNA template used is immature nuclear rRNA. Finally, reconstitution requires a 12 hours dialysis step from high salt to milder conditions at 23°C (Mangiarotti and Chiaberge, 1997).
These results suggest that, in contrast to prokaryotic ribosome assembly, *in vitro* eukaryotic ribosomal assembly is not a self-assembly process. This result seems to be in good agreement with the numerous essential ribosome biogenesis factors described in eukaryotes.

"The impression is that a government defense contractor was given a fully functional working prototype bacterial ribosome that was subsequently "reengineered" to do the same job, and was ultimately delivered at a cost of one third of the nation 's gross domestic product" (quoted from Williamson, 2003).

3.1.2.2. *In vivo* ribosomal protein assembly with rRNA and their involvement in rRNA maturation events.

Classical experiments were determining a rough kinetic of r-protein assembly with rRNA in different eukaryotic cells (review in Hadjiolov, 1984-85). These experiments were based on metabolic labelling followed or not by nuclear-cytoplasmic fractionation. The labelled ribosomal subunits were then separated by sucrose gradient and the amounts of radioactivity incorporated into the individual r-protein were determined.
These experiments suggest that eukaryotic r-proteins assemble into pre-ribosomes with different kinetics, thus accordingly they were classified as early or late assembling r-proteins (review in Hadjiolov, 1984-85).
In addition, immuno-localisation of one rpS and one rpL was performed using Miller's chromatin spreading technique, strongly suggesting that some r-proteins might assemble co–transcriptionally with the rRNA (Chooi and Leiby, 1981).

For instance this is probably the best picture of eukaryotes r-proteins *in vivo* assembly available.

At the beginning of this work little was known about the consequences of loss of function of individual r-proteins, on cell viability and ribosome biogenesis. In fact systematic deletions of the single copy genes coding for r-proteins in *S. cerevisiae* demonstrated that only 4 of the 19 r-proteins single copy genes are not essential for cell viability. Furthermore, for the r-proteins encoded by two copy genes in *S. cerevisiae*, only a few double knockout had been established, which were suggesting that these r-proteins are also essential for cell viability (see Moritz et al., 1990; Demianova et al., 1996; van Beekvelt et al., 2001a; Tabb-Massey et al., 2003).
In addition, haplo-insufficiency analyses in *S. cerevisiae*, and in other eukaryotes suggested that the expression level of a subset of r-proteins influences cell growth and development (Lucioli et al., 1988; Saeboe-Larssen et al., 1998).
One good example of the effect of r-protein haplo-insufficiency comes from the isolation of "minute" mutants in *Drosophila*. Such mutant flies display a dominant phenotype of slower development, short and thin bristles, reduced fertility and poor viability (Saeboe-Larssen et al., 1998). Many of these mutations were originally mapped within r-proteins genes. In fact recent systematic genome mapping of all the known minute mutants indicates that most (64/65 analysed) of the mutated genes are r-proteins encoding genes (Saeboe-Larssen et al., 1998, Marygold et al., 2007).

Whether these effects are related to ribosome biogenesis and/ or ribosome function defects or to extra-ribosomal functions of r-proteins remains to be determined.

The function of several individual r-proteins in ribosome biogenesis was examined.

One well-studied example is rpL10. It was shown that this r-protein interacts with Nmd3p. Interestingly, Nmd3p contain a functional NES which is recognised by the ribosomal exportin Crm1p/ Xpo1p (Ho and Johnson, 1999; Ho et al., 2000; Gadal et al., 2001). In addition, further analyses suggested that these factors are required for LSU export. Thus these results indicate that Nmd3p via its interaction mediated by rpL10 with pre-ribosomes can serve as an adapter for the export machinery.
In the light of recent results this model evolved slightly. In fact it seems that the LSU is exported throughout Crm1p/ Nmd3p interaction, and that rpL10 would associates in the

cytoplasm with the neo-exported LSU, thus allowing the release of Nmd3p from the LSU (Hedges et al., 2005; West et al., 2005).
Altogether, these results suggest that in the absence of rpL10 assembly, the export machinery and/ or adapter cannot be released from the pre-ribosome. Thus inducing an indirect ribosomal subunit export block throughout a factor titration mechanism as it has been suggested earlier for other factors (see Fromont-Racine et al., 2003; Lebreton et al., 2006).
It is not yet clear whether Nmd3p interacts directly with one or several r-proteins and/ or the rRNA to trigger LSU nuclear export.

Another well-studied example is the role of rpL5 in the assembly of the 5S rRNA with pre–60S particles. It was shown that rpL5 and 5S rRNA form a sub-complex required for 5S rRNA incorporation into pre-60S particles (Deshmukh et al., 1993). More recently it was suggested that a ribosome biogenesis factor mediates the interaction of another r-protein rpL11 with the rpL5-5S rRNA complex (Nariai et al., 2005). However the exact sequence of these assembly events remains to be determined.

Finally, with the help of conditional mutants of r-proteins from both subunits it was demonstrated that in some cases depletion of yeast r-proteins disturb rRNA maturation events most probably due to assembly defects or lack of rRNA folding (among others Rotenberg et al., 1988; Moritz et al., 1990; Moritz et al., 1991; Demianova et al., 1996; van Beekvelt et al., 2001a; Tabb-Massey et al., 2003).

3.2. Contribution of r-proteins to ribosome function.

From a structural and biochemical point of view the translation reaction can be seen as an RNA dependent process (see introduction 1.2.3.), which is summarised by the generally used term ***"the ribosome is a ribozyme"***. Despite clear evidence that r-proteins modulate the translation reaction this statement *"in the minds of many in the field has left ribosomal proteins in the dark as merely glue"* (quoted from Brodersen and Nissen, 2005).

In fact, *"apart from being RNA-glue …they indeed seem to have a social life after all"* (quoted from Brodersen and Nissen, 2005).

Although, peptide bond formation could be reconstituted *in vitro* by rounds of evolution and selection of short RNA (see Zhang and Cech, 1997; Zhang and Cech, 1998; Anderson et al., 2007). *"The ribosome is a ribozyme, admittedly one dependent on structural support from protein components-substantially deproteinized large subunits still carry out peptidyl transfer, although complete deproteinization destroys this reactivity (Noller et al., 1992)"* (quoted from Cech, 2000)

Indeed, efficient ribosome function depends on the cooperation between the rRNA and r–proteins. This cooperative nature is evident from different points of view and some examples are illustrating this point here after.

Many genetic screens, particularly in *E. coli* have identified mutations that confer resistance to various classes of antibiotics (Saltzman and Apirion, 1976; Dabbs, 1978; Gregory and Dahlberg, 1999). Interestingly, some of these mutations were described to affect r-proteins, despite no direct contact between the drug and the r-protein. Furthermore, mutations affecting r-proteins in one subunit can (1) be suppressed by mutations in other r-proteins from both ribosomal subunits (Dabbs, 1978), or (2) modulate the function of the ribosomal subunit in *trans*, or (3) induce structural rearrangements in both ribosomal subunits (e.g. Davies et al., 1998).

For instance, many results suggest that in both pro- and eukaryotes r-proteins contribute to many if not all aspects of ribosome function like translation fidelity, interaction with mRNA and translation associated factors (reviewed in Brodersen and Nissen, 2005; Wilson and Nierhaus, 2005).
Furthermore, r-proteins play a crucial role in the recruitment of external factors required for example in protein targeting or for co-translational protein folding. It was demonstrated that the r-proteins localised at the large subunit exit-tunnel serve as anchor points for functionally important interactions with the signal recognition particle, ER membrane-embedded sec complex and trigger the recruitment of factors of the chaperone system (see among others Beckmann et al., 2001; Kramer et al., 2002; Gu et al., 2003).

In addition, r-proteins are known to be targets of various post-translational modifications (Kruiswijk et al., 1978a; Kruiswijk et al., 1978b; Arnold and Reilly, 1999; Arnold and Reilly, 2002) that can putatively influence the ribosome function.

Noteworthy, in eukaryotes, r-proteins can be encoded by multiple genes. In *Arabidopsis thaliana*, each r-protein is encoded on average by four homologues genes, many isoforms of which have been shown to be present in the 80S ribosome (Giavalisco et al., 2005). In *Dictyostelium discoideum*, the composition of the ribosome has been shown to change depending of the developmental stage, both in qualitative and quantitative terms as well as in terms of modifications (Giavalisco et al., 2005).

A so-called "ribosome filter hypothesis" has been suggested to explain the function of such heterogeneity within the ribosome population (Mauro and Edelman, 2002). This model proposes that a particular set of ribosomal proteins and/ or rRNA could favour the translation of specific mRNAs. In this respect, the ribosome composition becomes an important player for the understanding of protein expression regulation.

Further work needs to be done in order to support this model, but such a scenario would add another layer to the importance of r-proteins in the regulation of gene expression.

3.3. Extra-ribosomal function of r-proteins.

Several studies finally challenge the simple view that r-proteins are "only" ribosome constituents pointing towards extra-ribosomal function of r-proteins in replication, transcription, DNA repair… (reviewed in Wool, 1996)

It was shown, in both pro- and eukaryotes, that some r-proteins have the ability to bind their own mRNA transcripts, thus decreasing the production of this protein (Vilardell and Warner, 1994; Li et al., 1996; Vilardell and Warner, 1997; Fewell and Woolford, 1999; Nomura, 1999; Vilardell et al., 2000; Badis et al., 2004; Nierhaus, 2004). R-protein dependent autogenous control was proposed to control stoichiometric production of r-proteins and to ensure coordinated regulation of rRNA and r-proteins synthesis especially in *E. coli* (see for reviews Nomura, 1999; Nomura, 2001; Nierhaus, 2004).

Recently, it was demontrated in higher eukaryotes that a r-protein can bind to a mRNA and consequently inhibit its translation (Mazumder et al., 2003; Zimmermann, 2003). These results offer new perspectives in term of gene expression control upon environmental changes.

Ribosome production is a high energy consuming process therefore ribosome biogenesis needs to be coordinated with cell growth and proliferation.

It was shown in higher eukaryotes that following conditions inducing so-called “nucleolar stress” or “ribosomal stress” ribosome biogenesis is impaired, thus leading to an increase of free r-proteins. At least four of these free r-proteins were shown to bind to MDM2, thus blocking MDM2-mediated p53 ubiquitination and degradation, and inducing a p53 dependent cell cycle arrest (see Chen et al., 2007; Lindstrom et al., 2007).

These results suggest a possible extra-ribosomal function of r-proteins in the coordinated regulation between ribosome biogenesis and cell proliferation.

4. Aims of this work: *in vivo* functions and assembly pathway of SSU r–proteins in eukaryotes.

Coding and non-coding RNPs occupy a pivotal role in cellular metabolism. During the last decades the number of identified complexes has grown extensively. Among them are the ribosomes, tRNA and mRNA containing complexes, snRNPs, SRP, telomerase, snoRNPs, scaRNPs and miRNPs …
All these complexes share similarities in their biogenesis: proteins have to be assembled onto the RNA transcript, the RNA of most of these molecular complexes has to be processed and/ or modified and finally functional complexes have to be delivered to their place of function.

The small ribosomal subunit (SSU) is such a RNP, and since in eukaryotic cells its biogenesis is very complex as well as temporally and spatially organised, it represents a valid model to understand many facets underlying (eukaryotic) RNPs biogenesis. The eukaryotic SSU contains around 32 r-proteins and one rRNA. Despite many efforts, the *in vivo* roles of many eukaryotic SSU r-proteins in ribosome biogenesis and function and how they assemble with the rRNA to form a functional ribosomal subunit remain unclear.

To get a comprehensive insight into the *in vivo* function of r-proteins in ribosomal subunit biogenesis and ribosome function, a systematic analysis was performed using the model organism *Saccharomyces cerevisiae* with the following objectives:
(1) to establish a system to analyse systematically the consequences of r-protein depletion in ribosome biogenesis and function.
(2) to use this system in order to determine the primary function of individual r-proteins in ribosome biogenesis.
(3) to establish tools allowing to monitor the *in vivo* assembly status of individual r-proteins in wildtype and mutant cells.
(4) and to determine the *in vivo* r-protein composition of functionally relevant assembly intermediates.

These tools and approaches should allow to draw correlations between RNP maturation, assembly and cellular localisation, which is crucial to understand the coordination and regulation of RNP biogenesis.

RESULTS

1. Function of SSU r-proteins in ribosome biogenesis.

In order to understand the function of each individual SSU r-proteins in SSU biogenesis, a systematic functional study of yeast SSU r-proteins was performed. For this purpose strains conditionaly expressing SSU r-proteins were systematically constructed (results 1.1.). The consequences of loss of function of individual SSU r-proteins on ribosome biogenesis and function were analysed and are described below (see results 1. and 3. respectively).

1.1. Most of the SSU r-proteins are required for yeast cell growth.

1.1.1. Construction of yeast strains deleted in the SSU r-protein genes.

Since, in yeast, many SSU r-proteins are expressed from nearly identical duplicated genes, the invalidation of SSU r-proteins requires in most cases the knockout of two higly similar copies genes present in the haploid genome (Fig. 19). Diploid strains were generated by mating two yeast strains in which either one of the two ribosomal gene copies was replaced by a different selection marker (KanMX4 replaced by HIS3 or HIS3MX6). The resulting diploid (*rpsXA*::KanMX4/RPSXA, *rpsXB*::HIS3/RPSXB) and diploids for the single copy genes (*rpsY*::KanMX4/RPSY) were transformed with a vector expressing a wildtype copy of the respective SSU r-protein (pRPSX; URA3 see materials & methods 6.). Positive transformants were incubated on sporulation media, and subjected to tetrad analysis. Depending on the case (single or duplicated genes), haploid strains bearing deletions in one/ or both rpS gene copies were selected ($G418^R$ for Δ*RPSY* or $G418^R/HIS^+$ for Δ*RPSXA*, Δ*RPSXB*).

Viability of complete knockout strains was then tested on 5-FOA containing plates (see materials & methods 1.2.1.4.). Twenty-six of the 28 SSU r-proteins studied herein are FOA^S indicating that the plasmid expressing one of the two r-proteins variants can not be lost and thus these SSU r-proteins are required for cell growth. Of the remaining four SSU r–proteins no clear knockout could be constructed, these four strains are still under investigation. However, two from these remaining SSU r-proteins, rpS4 (Synetos et al., 1992)

and rpS21 (Tabb-Massey et al., 2003) were already reported to be essential for cell growth (see also www.yeastgenome.org).

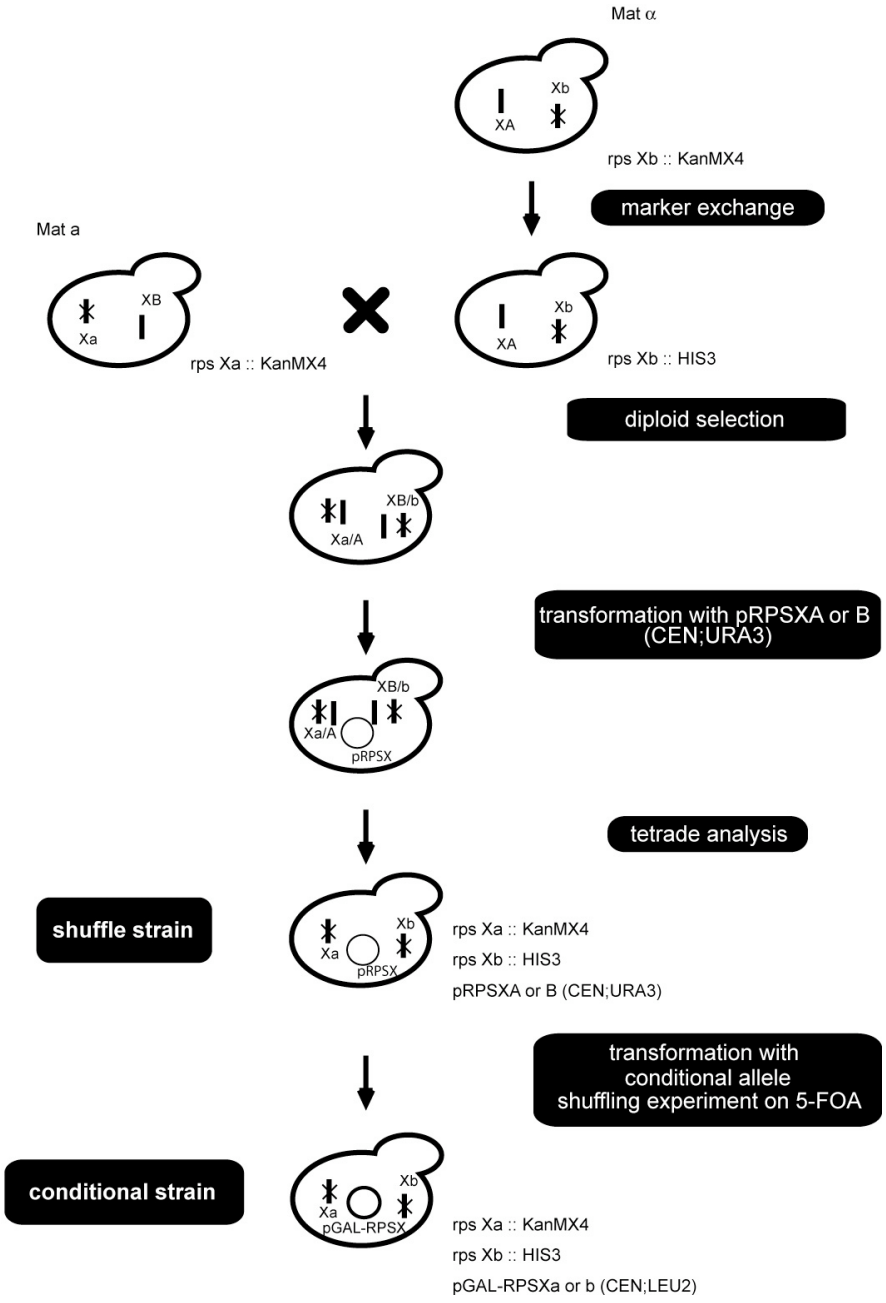


Figure 19: Generation of a yeast-strain collection in which (duplicated) genes coding for ribosomal proteins are deleted.
A strategy to derive knockout strains and strains conditionally expressing r-proteins is depicted.
KanMX4 deletion markers of knockout strains were replaced by HIS3 or HIS3MX6 markers. Haploid strains bearing either a KanMX4 or a HIS3/HIS3MX6 marker in one of the two genes coding for the same r-protein copies were crossed. The resulting diploid cells were transformed with a URA3 plasmid encoding a wildtype gene copy of r-protein X (RPSX). Strains were sporulated, submitted to tetrade analysis, and selected for both markers (His^+ and $G418^R$) to derive the corresponding shuffle strain for gene RPSX. Any conditional strains can be obtained by exchange of the shuffle plasmid with a plasmid expressing a respective mutant allele, p*rpsXa*.

Furthermore, viability of all knockouts could be rescued expressing one of the two r-proteins variants, indicating that the essential function of the respective duplicated r-protein is redundant. This observation is in agreement with the systematic single-knockout studies, where one of the two gene copies was deleted, and the remaining copy gene was able to support cell growth (Winzeler et al., 1999; Giaever et al., 2002).

From the two non essential SSU r-proteins namely rpS12 and rpS25, the single copy gene encoding rpS12 was previously shown to be not essential for cell viability and was obtained from Euroscarf. Interestingly, haploid strains bearing a complete knockout of the two genes encoding for rpS25A/B obtained after tetrad analysis were FOA^R suggesting that this r-protein is dispensable for cell growth. In order to confirm this result, the genotype of this strain was confirmed by PCR analysis (see Fig. 20). Together, these results demonstrate that the eukaryote specific rpS25, encoded by 2 closely related genes, is not essential for cell growth in the condition tested.

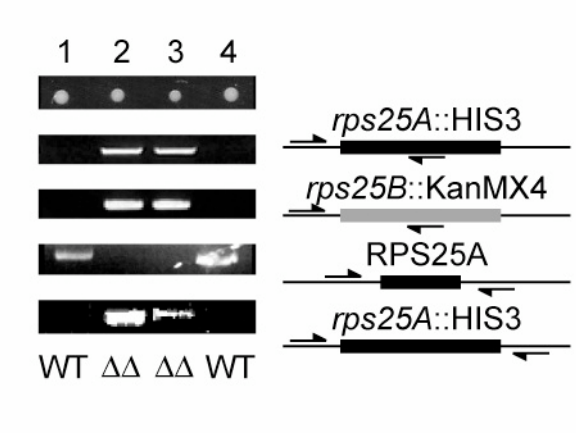


Figure 20: The rpS25 isoformes are not essential for cell growth.
Heterozygous diploid cells for the RPS25 genes were sporulated (RPS25A/*rps25A*::HIS3; RPS25B/*rps25B*::KanMX4). Tetrads containing four viable spores were always obtained. Full RPS25 knockout clones (*rps25A*::HIS3; *rps25B*::KanMX4) were selected on selective media and genotypes were further confirmed by PCR using the primer combinations indicated in the figure.

1.1.2. Construction of yeast strains conditionally expressing SSU r-proteins.

Strains conditionally expressing the respective SSU r-protein were generated by exchanging the plasmid containing the wildtype copy with a vector carrying a conditional allele (see Fig.19 and material & methods 6.2.2.). The tightly regulated GAL1 promoter, yielding high gene expression levels in the presence of galactose and being strongly repressed in presence of glucose was used to control SSU r-protein gene expression.

Viability of all knockouts could be rescued by over-expression of one of the two r-proteins alleles. In addition, these conditional alleles contain only the coding sequence of the respective yeast SSU r-protein, although many of the chromosomal SSU r-protein genes contain intron sequences. Interestingly, growth complementation by the intronless alleles suggests that intron sequences and splicing reaction within SSU r-protein mRNA play no essential role for cellular processes including maturation and transport of the respective mRNA.

Finally, SSU RPS gene expression was shutdown by transfer of the strains onto glucose containing media. In all the cases, cell growth was inhibited, which is in agreement with the viability test on 5-FOA-plates, with only one exception: knockout of RPS31 was lethal on 5-FOA-plates, whereas shutting down gene expression of RPS31 on glucose resulted in a strong retarded growth phenotype (see Fig. 21A).

In order to determine whether the strong retarded growth phenotype observed after depletion of RPS31 was due to a leaky expression of this gene and to demonstrate the specificity of the GAL1 promoter dependent gene expression regulation Northern blot experiments were performed.

After 2 and 4 hours transfer to glucose containing media, the mRNA levels of the GAL1 controlled RPS genes tested, including rpS31-mRNA, could not be detected, whereas the endogenous rpS-mRNA levels were unaffected, demonstrating the specific glucose-dependent down regulation of GAL1 controlled RPS gene expression (see Fig. 21B).

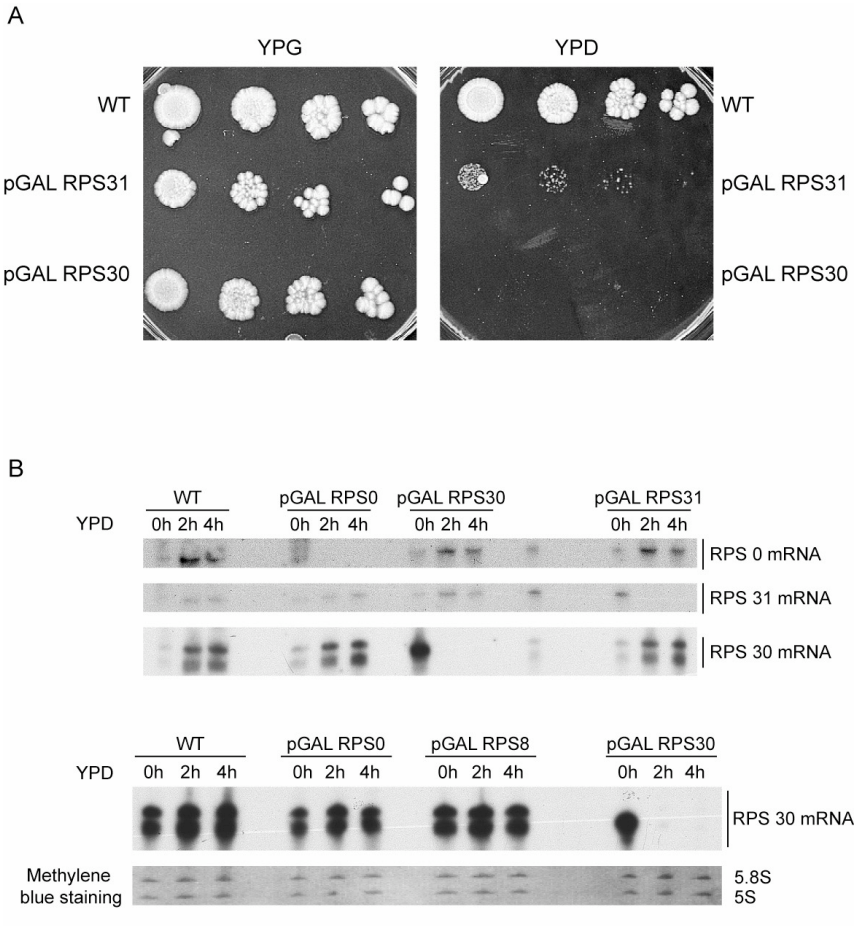


Figure 21: RPS gene shutdown impairs cell growth and is specific.

(A) Depletion of SSU r-proteins strongly inhibits cell growth.
Serial dilutions of wildtype cells and strains conditionally expressing the RPS30 and RPS31 genes were plated on solid full media containing either glucose or galactose as a carbon source and incubated at 30°C for 5 days.
(B) Depletion of a single r-protein does not influence the mRNA level of other r-proteins.
Various strains conditionally expressing single r-proteins were grown on galactose (0 h) or in presence of glucose (2 h and 4 h) in order to shutdown the expression of the corresponding r-protein.
Same amounts of total RNA were separated by PAGE-UREA, and transfered onto a nylon membrane as described in the materials & methods 2.2.5. and 2.2.6.3. Prominent rRNA species were stained with methylene blue and assigned according to their apparent running sizes. The different mRNA species were detected by hybridisation of the membrane with specific ^{32}P radiolabelled oligo probes for RPS0, RPS30 and RPS31 mRNA (see materials & methods Table 4). Signals were detected and visualised using a Phosphoimager as descrided in the materials & methods 2.2.8.

1.2. Most of the SSU r-proteins are required for synthesis of mature 18S rRNA.

Since most SSU r-proteins are required for cell growth, strains conditionally expressing SSU r-proteins were used to determine which of the essential r-proteins are required for the production of the mature SSU.

The dynamics of rRNA synthesis were analysed after shut down of SSU r-protein gene expression in conditional mutants by [^{3}H] uracil pulse analysis (see Fig. 22).

Newly synthesised levels of both mature 18S rRNA and 25S rRNA were significantly reduced; however, reduction in 18S rRNA synthesis was predominant. Only two exceptions were observed: shut down of RPS30 and RPS8 gene expression affected production of both 18S and 25S mature rRNA to a similar extent, suggesting that both ribosomal subunits are still produced at a comparable low level.

Since, the two ribosomal subunits are formed from a common 35S pre-rRNA limiting the amounts of any of the SSU r-proteins interferes with efficient production of both mature ribosomal subunits. It is a general observation in mutants affecting SSU production that processing of the LSU rRNA still proceeds, however with reduced amount and/ or kinetics. However, because reduction in 18S rRNA synthesis is predominant, the influence of the SSU r-proteins on proper maturation of the SSU is more direct.

Even though significantly reduced, levels of newly synthesised 18S rRNA varies in the different conditional strains (from nearly 0% to 7% of the wildtype production). After depletion of the SSU r-proteins rpS7, rpS10, rpS30 as well as rpS31 more than 4% of the wildtype 18S rRNA is still generated, indicating that SSU biosynthesis is not completely blocked in these strains.

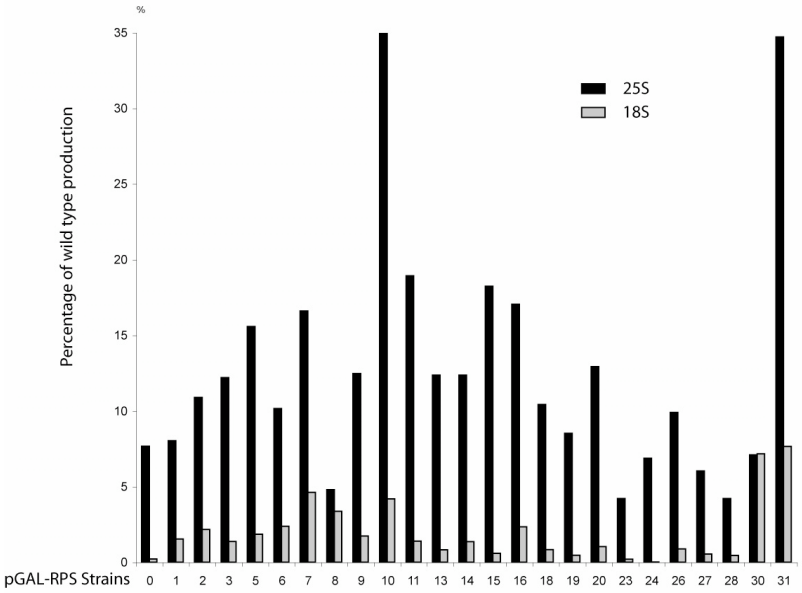


Figure 22: Effect of SSU r-protein depletion on newly synthesised rRNA.

Yeast cells were shifted to glucose containing medium for 2 h to deplete rpS synthesis and were pulse labelled for 15 min with [^{3}H] uracil. RNAs were isolated and separated by gel electrophoresis and blotted onto a nylon membrane. Membrane slices containing ^{3}H-labelled 25S and 18S rRNA, respectively, were cut, and incorporation of [^{3}H] uracil activities were determined in a scintillation counter. Background activity was subtracted, and total activity was normalised according to steady-state 25S rRNA (as determined by Northern blotting analysis) in the load. The amount of 25S and 18S rRNA in mutant strains is compared with wildtype conditions. See also materials & methods 4.1.

In strains lacking the non-essential SSU r-proteins rpS12 and rpS25, similar amounts of mature 18S and 25S rRNA were produced, however, at a reduced level compared to a wildtype strain: 65% and 30%, respectively (data not shown). These effects on rRNA synthesis are in good agreement with the slow growth phenotype observed in these strains.

These data clearly suggest that most SSU r-proteins being essential for growth are also required for the proper production of mature SSU. Thus, SSU r-proteins can either play a direct role in the maturation pathway or their presence might be mandatory to ensure that only properly assembled ribosomal subunits accumulate in the cytoplasm.

1.3. SSU r-proteins are required at different steps of the SSU biogenesis.

Since, SSU r-proteins are required for formation of mature 18S rRNA, this suggests that processing and/ or production of rRNA precursors is impaired after depletion of the SSU r-proteins.
However, it is not clear whether depletion of any of the SSU r-proteins inhibits the same or different steps in the rRNA processing pathway.
Therefore, steady-state levels of rRNA intermediates leading to formation of mature 18S rRNA were analysed after depletion of single SSU r-proteins (Fig. 23). Transblotted rRNA species were detected by using specific probes (see Fig. 23). According to their different RNA processing patterns, strains could be divided into six different groups and subgroups (see summary Fig. 24 and sections below), demonstrating that almost all-essential SSU r-proteins are involved in the control of distinct maturation steps.
Strains were first classified into two main groups as described below:

(I) 20S rRNA is not detectable after depletion of the respective SSU r-proteins (group I, Fig. 24B and results 1.3.1.).

(II) 20S rRNA is produced with either wildtype or slightly reduced efficiency (group II, Fig. 24B and results 1.3.2. and following section).

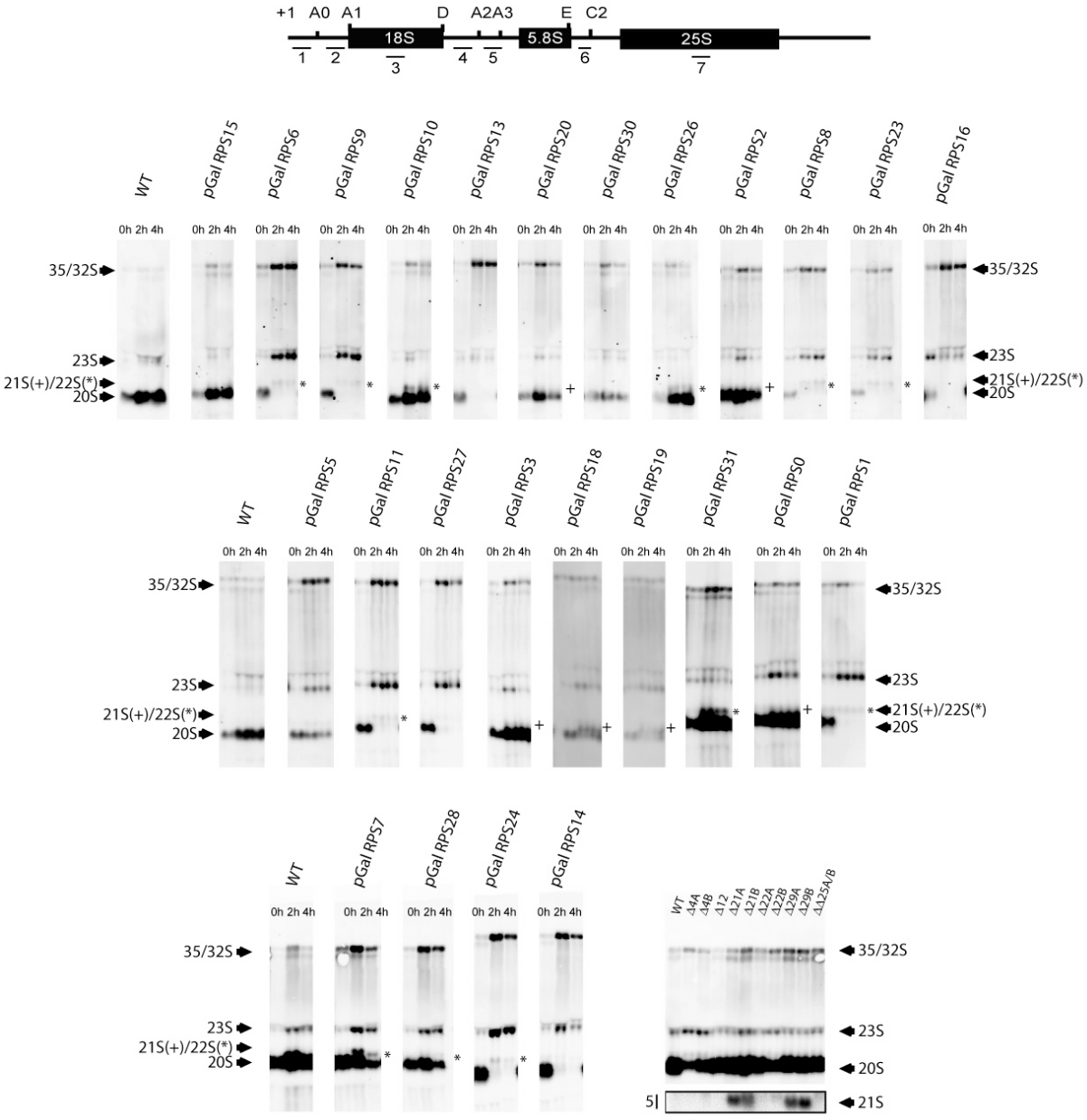


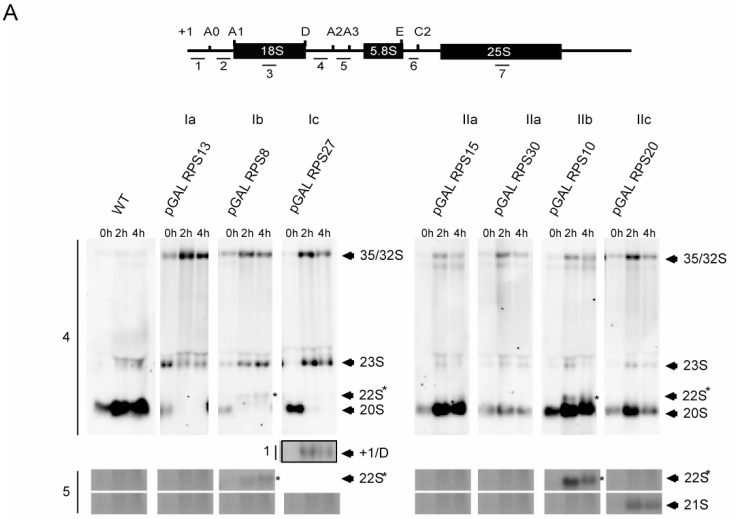
Figure 23: R-proteins control distinct pre-rRNA processing steps.

Analysis of pre-rRNA processing in strains depleted in r-proteins by Northern blot. RNA from the same amount of cells was extracted from either wildtype or GAL-RPS strains grown in YP-galactose (0 h) or after 2 h and 4 h shift to YP-glucose.
Probes 1–7 (see materials & methods table 4) were used to map the different rRNA species. The results for probes (4) ITS1 and (5) are shown.
The plus sign (+) and asterisk (*) indicate the detection of 21S and 22S rRNA, respectively. The presence of 21S and 22S rRNA intermediates was further confirmed with probes (2) and (5).

1.3.1. Depletion of a subset of SSU r-proteins does not allow detectable production of 20S rRNA.

Mutant strains in which 20S rRNA is not detectable accumulate 35S and 23S rRNA (Fig. 23-24). Elevated levels of 35S rRNA indicate that cleavage at sites A0, A1, A2, and A3 is delayed. When cleavage at site A3 precedes processing at sites A0, A1, and A2, 23S rRNA is generated and represents either an aberrant RNA intermediate that is normally degraded (Venema and Tollervey, 1995) or an intermediate of an alternative pathway to mature 18S rRNA (Granneman and Baserga, 2004 see also introduction 2.2.2.3.). Accordingly, accumulation of the 23S rRNA indicates that the respective depleted SSU r-protein is involved in early cleavage steps at sites A0, A1, and A2.

A



B

Group	I			II		
Phenotyp	20S is not detectable			20S is made		
Sub-Group	a	b	c	a	b	c
rRNA species accumulating after depletion	35S/23S A0,A1,A2,D inhibited A3 delay	35S/23S/22S A1,A2,D inhibited A0 delay	35S/23S/+1-D A0,A1,A2, inhibited A3 delay; D ? can occur with delay	35S/23S/20S A0,A1,A2,A3 delay	35S/23S/22S A0,A1,A2,A3 delay	35S/23S/21S A0,A1,A2,A3 delay
RPS	RPS13 RPS14 RPS16	RPS1 RPS6 RPS8 RPS9 RPS11 RPS23 RPS24	RPS27	RPS5 RPS15 RPS30	RPS7 RPS10 RPS26 RPS28 RPS31	RPS0 RPS2 RPS3 RPS18 RPS19 RPS20

Figure 24: Summary of the different rRNA processing defects induced after SSU r-protein depletion.

(A) An overview of representative strains resulting in a similar processing pattern is depicted. Northern blot hybridised with probe ITS1 (4, upper) is shown. Detection of 21S/ 22S rRNA using probes A2/A3 (5) and fragment +1–D rRNA using probe +1-A0 (1) is shown (lower) (see Table 4)

(B) Classification of r-proteins according to their pre-rRNA processing defects. Ribosomal proteins are sorted into group I if their depletion results in loss of 20S rRNA. If 20S rRNA is still produced, they belong to group II. Subgroups were classified according to the presence or absence of 21S, 22S, or +1-D rRNA species (Ia, Ib, or Ic and IIa, IIb, or IIc, respectively).

Two further subgroups in which 35S, 23S rRNAs are accumulated can be distinguished, one in which cleavages at A0, A1, and A2 are equally inhibited (group Ia) and one in which cleavage at A0 is affected but occurs at a low rate to yield 22S rRNA (group Ib) (see Fig. 24B).

Depletion of rpS27 resulted in a comparable 35S and 23S rRNA accumulation observed for group Ia. However, in this strain, a rRNA intermediate was also detected with probes specific for the region upstream of A0 and upstream of the 3' boundary of 18S rRNA, whereas probes complementary to ITS1 did not recognise this rRNA intermediate (Fig. 25A). In this mutant strain, cleavage at sites A0, A1, A2 is inhibited, whereas processing at site A3 is delayed, leading to a pronounced level of 23S rRNA. Apparently, in this mutant, a minor fraction of rRNA is cleaved at the 3' boundary of the 18S rRNA (site D?) bypassing cleavage at A2 while cleavage at A0, and A1 does not occur resulting in an intermediate slightly larger than the 22S (+1 to D?). A similar fragment spanning the region from +1 to site D was recently described (Kufel et al., 2003; Vos et al., 2004).

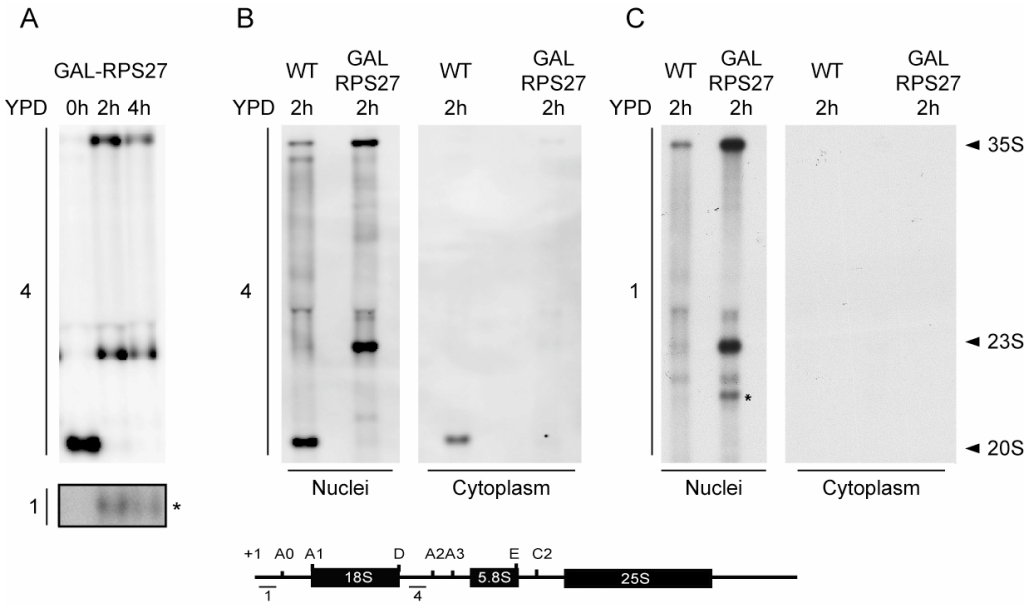


Figure 25: Depletion of rpS27 induces the formation of a non-canonical nuclear rRNA intermediate.

(A) Analysis of pre-rRNA processing in strain depleted in rpS27 by Northern blot. RNA from the same number of cells was extracted from GAL-RPS27 strain grown in YP-galactose (0 h) or after a 2 h or 4 h shift to YP-glucose. Probes 1–7 (see Table 4) were used to map the different rRNA species. The results for probes (4) ITS1 and (1) are shown. The asterisk (*) indicates the detection of a +1/D? rRNA specie.

(B) and (C) The +1/D? rRNA fragment is detected in the nuclear fraction. Wildtype and GAL-RPS27 strains were incubated for 2 h in YP-glucose media. Nuclei and cytoplasm were further fractionated as described in materials & methods 4.3. RNA was extracted and separated by gel electrophoresis and blotted. The membrane was then hybridised by probe (4) and (1) shown in (B) and (C) respectively. The asterisk (*) indicates the detection of a +1/D? rRNA species.

Since, in wildtype cells cleavage at site D occurs in the cytoplasm, but A0, A1, A2 cleavages are nuclear processing events, nuclear-cytoplasmic cell fractionations were carried out after depletion of RPS27 in order to localise this rRNA species. Interestingly, the +1-D? fragment observed after depletion of RPS27 appears to be mainly located in the nucleus (see Fig. 25B-C). This result suggests that rRNA cleavage in the ITS1 region (between D-A2) can happen in the nucleus. However, exact 5' and 3' end RNA mapping needs to be performed in order to determine the exact cleavage site(s) for this rRNA species. Furthermore, complete or partial (re)localisation to the nucleus of the known cytoplasmic ribosome biogenesis factors required for D site cleavage in this condition needs to be investigated.

1.3.2. Depletion of a subset of SSU r-proteins still allows detectable production of 20S rRNA.

As indicated earlier (Fig. 23-24) depletion of a subset of SSU r-proteins still allows production of 20S rRNA. In wildtype cells, 20S rRNA occurs after processing of 35S/(23S) pre-rRNA at sites A0, A1, and A2 (see introduction 2.2.2.3. and Fig. 11). However, both, the slightly elevated levels of 35S and 32S rRNA and the detectable amounts of 23S rRNA in the same mutants indicate that cleavage at sites A0, A1, and A2 is either delayed or not as efficient as in wildtype yeast. Three subgroups of mutant strains producing 20S rRNA could further be distinguished (Fig. 24B): strains that accumulate either 21S rRNA (like pGAL-RPS20), 22S rRNA (like pGAL-RPS10), or none of these two species (like pGAL-RPS15). Appearance of 22S rRNA indicates cleavage at site A0 but defective processing at sites A1 and A2, whereas 21S rRNA results from inefficient cleavage at site A2 and cleavage occurring at sites A0 and A1 (see introduction 2.2.2.3. and Fig. 11). These results show that a distinct subset of SSU r-proteins supports each cleavage step leading to 20S rRNA, the export-competent form of pre-18S rRNA. Although 20S rRNA is made in these strains, its steady-state amount is more or less reduced compared to wildtype cells.

1.4. A subset of SSU r-proteins is required for efficient pre-40S subunit export.

Since, 20S pre-rRNA is the direct substrate for late nuclear and cytoplasmic maturation events, there are several possibilities regarding the function of SSU r-proteins of group II in SSU biogenesis. Mutants still producing 20S rRNA after depletion of individual SSU r–proteins (1) are directly impaired in export of 20S rRNA, (2) are inhibited in making

20S rRNA competent for export from the nucleus to the cytoplasm, or (3) are deficient in the cytoplasmic processing steps. To differentiate between these possibilities, dynamic and steady state maturation assays were applied in order to distinguish spatially and temporally the maturation state of 20S rRNA containing pre-SSU´s produced after depletion of this subset SSU r-proteins.

1.4.1. Nuclear-cytoplasmic cell fractionation.

Neo-synthesised RNA in cells depleted of individual SSU r-proteins were pulse-labelled with [^{3}H] uracil. Subsequent cell fractionation was performed to localise neo-synthesised rRNA species (see material & methods 4.3.).

Results presented in figure 26 show that strains having a low amount of steady-state 20S rRNA after depletion of SSU r-proteins (rpS5, rpS18, and rpS19) (see Fig. 23 and 26B) contain only a minor amount of neo-synthesised 20S rRNA in nuclei and no detectable 20S rRNA in the cytoplasmic fraction (Fig. 26A). This suggests that 20S rRNA cannot reach the cytoplasm because either it is unstable, and/ or it is exported at such a reduced level that it cannot be detected.

Interestingly, in conditional mutant strains yielding higher levels of steady-state 20S rRNA (strains depleted in rpS0, rpS2, rpS3, rpS7, rpS10, rpS15, rpS20, rpS26, rpS30, or rpS31 see Fig. 23), similar amounts of neo-synthesised 20S rRNA levels compared to wildtype cells (between 50% and 110%) are found in nuclear fractions (Fig. 26A).

Surprisingly, in strains depleted in rpS0, rpS2, rpS3, rpS10, rpS15, or rpS26, the amount of neo-synthesised 20S rRNA in the nuclear fractions is similar to wildtype levels, but the amount of newly made 20S rRNA reaching the cytoplasm during the 15 min of pulse is strongly reduced (see Fig. 26A).

In contrast, when one of the SSU r-proteins rpS7, rpS20, rpS30, or rpS31 is lacking, a significant amount of neo-synthesised 20S rRNA reaches the cytoplasm. Furthermore, in strains depleted in rpS7, rpS30, and rpS31, a significant amount of the cytoplasmic 20S rRNA can be further processed to 18S rRNA (see Fig. 26A).

Accordingly, r-proteins rpS7, rpS30, and rpS31 seem to be not strictly required for rRNA maturation and nuclear export, indicating that their primary role might be in protein translation (see results 3.). On the other hand, these results suggest that export of pre–18S rRNA from the nucleus is inhibited in strains depleted in rpS5, rpS18, and rpS19,

while pre-18S rRNA export from the nucleus is clearly retarded in strains depleted in rpS0, rpS2, rpS3, rpS10, rpS15 or rpS26, but not completely inhibited.

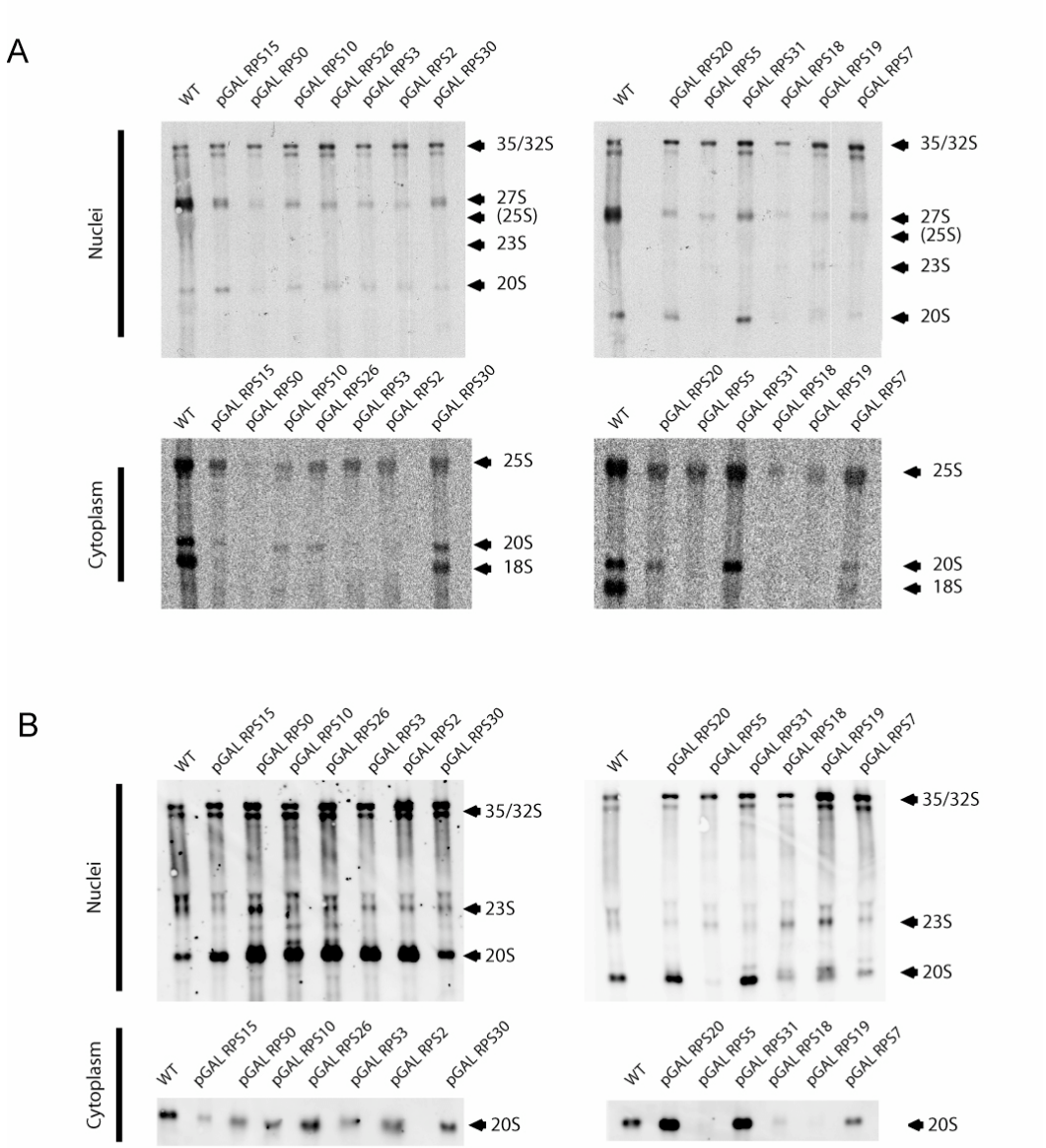


Figure 26: R-proteins control competence and efficiency of pre-18S rRNA export.

Nuclear export of 20S rRNA was analysed in GAL-RPS strains 2 h after transfer into YP-glucose.

(A) Analysis of newly synthesised pre-18S rRNA nuclear export by metabolic labelling and cell fractionation. After a 2 h shift to glucose containing medium, spheroplasts were labelled with [^{3}H] uracil for 15 min and fractionated in nuclei and cytoplasm. RNAs were isolated and steady-state amounts of 25S rRNA were determined by dot blot analysis. Equal amounts of steady-state 25S rRNA of cytoplasmic and nuclear fractions, respectively, were separated by gel electrophoresis and blotted. Newly synthesised ^{3}H-labelled RNAs were detected by phosphorimaging and quantified using Image Gauge software (Fuji).

(B) Steady-state analysis of rRNA in nuclei and cytoplasm.

The same blots as in (A) were developed using digoxigenin-labelled ITS1-probe 4 (Note that the cytoplasmic fraction of wildtype strains contained about 80%–90% of total 20S rRNA). 20S rRNA was quantified with the Fuji imaging system LAS3000, applying the AIDA software (Raytest).

Using a probe directed against the ITS1 region, steady-state levels of pre-18S rRNA in nuclear and cytoplasmic fraction from the blot used in Fig. 26A were determined.

No cytoplasmic steady-state 20S rRNA was detected in strains lacking rpS5, rpS18, or rpS19, while a low but detectable amount of steady-state 20S rRNA can be observed in the nuclear fraction. This result confirms the role of rpS5, rpS18, and rpS19 in late nuclear pre-40S maturation step before the passage of 20S rRNA through the nuclear pore.

Mutants having a retarded nuclear export rate (rpS0, rpS2, rpS3, rpS10, rpS15 and rpS26) accumulate steady-state 20S rRNA in the nucleus. Surprisingly, cytoplasmic 20S rRNA of these mutants is rather stable, as it apparently accumulates over time and —after two hours depletion —it reaches levels between 40% and 120% of the cytoplasmic 20S rRNA intermediate in wildtype cells (in wildtype cells about 80% of 20S rRNA is found in the cytoplasm).

This result confirms the role of rpS0, rpS2, rpS3, rpS10, rpS15 and rpS26 to allow efficient passage of pre-40S through the nuclear pore, and also demonstrates the requirement of these SSU r-proteins in late cytoplasmic maturation of 20S into 18S rRNA.

1.4.2. Fluorescence in situ hybridisation of pre-rRNA.

In order to confirm the results described above **f**luorescent ***in situ*** **h**ybridisation (**FISH**) analyses using a Cy3 (cyanine 3) modified ITS1 probe were performed, allowing to verify the steady-state distribution of 20S rRNA and its precursors in the nuclei and cytoplasm of the mutants (Fig. 27).

Exclusively nucleolar and nuclear localisation of rRNA intermediates hybridising with ITS1 was visible in mutants depleted in rpS5, rpS18, and rpS19.

In mutants that are able to export 20S rRNA at a similar rate as wildtype cells (strains depleted in rpS20, rpS30, and rpS31) or that can accumulate 20S rRNA over time (strains depleted in rpS0, rpS2, rpS3, rpS10, rpS15 and rpS26), nuclear and cytoplasmic FISH signals were similar or only slightly reduced to those obtained with wildtype cells.

Although the dynamic range of the FISH analysis seems to be weaker, this result confirms the presence of exported 20S rRNA precursor to the cytoplasm in strains depleted in rpS0, rpS2, rpS3, rpS7, rpS10, rpS15, rpS20, rpS26, rpS30, or rpS31.

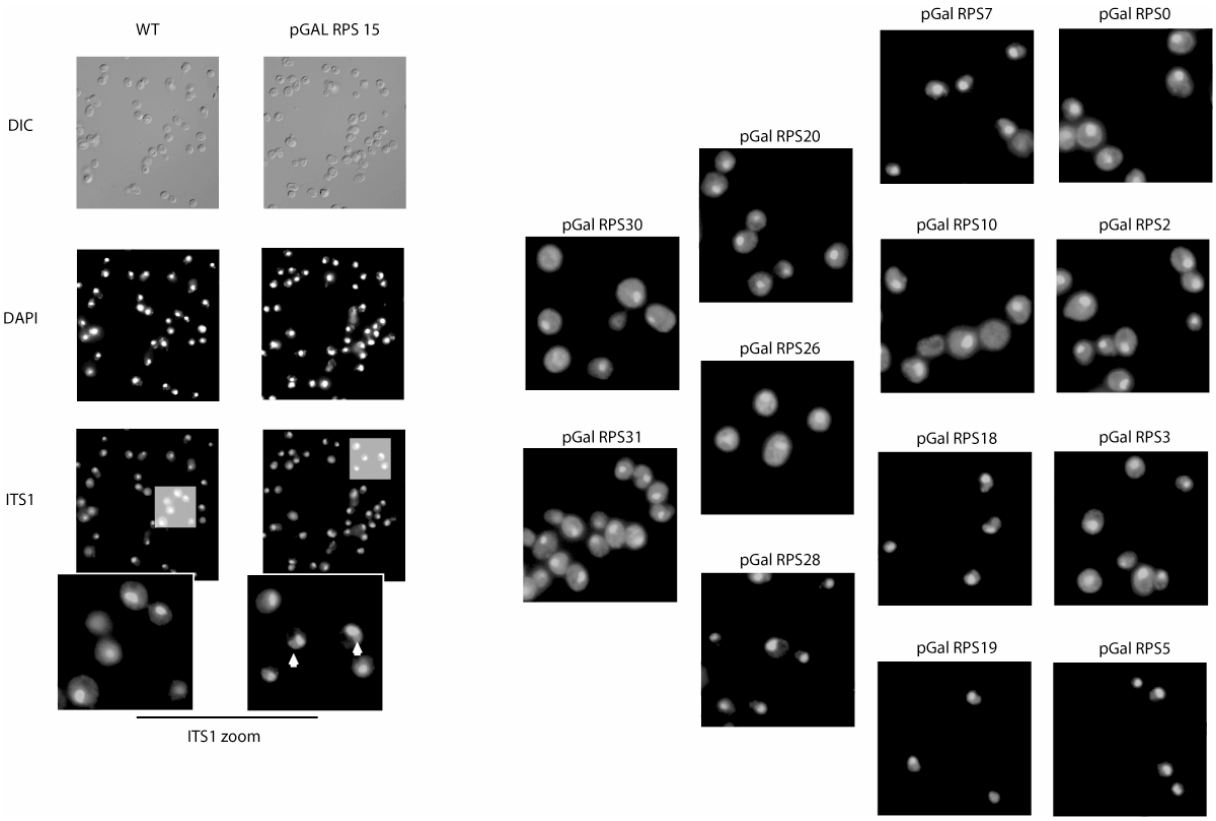


Figure 27: R-proteins control pre-18S rRNA export.
Nuclear export of 20S rRNA was analysed in GAL-RPS strains 2 h after transfer into YP-glucose by using FISH of pre-18S rRNA. A probe complementary to the D-A2 segment of ITS1 was used to localise pre-18S rRNA after depletion of rpS expression. Arrowheads indicate the nucleoplasm that was visualised by DAPI staining.

1.5. Summary on the function of SSU r-proteins in ribosome biogenesis.

In conclusion (see also summary Fig. 28), these results indicate that most individual SSU r-proteins are required (1) for efficient production of mature 18S rRNA (2) for different steps in SSU biogenesis, and can be grouped according to their rRNA processing defect phenotype after depletion. Interestingly, most of the known pre-rRNA intermediates described in the literature in wildtype and ribosome biogenesis mutant strains could be seen after depletion of individual SSU r-proteins, suggesting a crucial role of individual SSU r-proteins at every steps of the SSU maturation. The results indicate that SSU r-proteins can be further grouped according to the 20S rRNA maturation defects shown after depletion. (3) SSU r-proteins rpS5, rpS18, and rpS19 are required for late nuclear processing and/ or quality control steps leading to nuclear retention of 20S rRNA. (4) Depletion of SSU r-proteins rpS0, rpS2, rpS3, rpS10, rpS15 and rpS26 appear to first affect the export rate/ competence of the SSU and then the late maturation steps of conversion of 20S pre-rRNA into mature 18S rRNA. Furthermore,

(5) when one of the SSU r-proteins rpS7, rpS20, rpS30, or rpS31 is lacking, a significant amount of neo-synthesised 20S rRNA reaches the cytoplasm. Finally, (6) in strains depleted in rpS7, rpS30, and rpS31, significant amounts of the cytoplasmic 20S rRNA can be further processed into mature 18S rRNA.

Group	I			II		III			IV	
Phenotyp	**20S is not detectable**			**20S is made and not exported**		**20S is made and exported or retarded but not converted into 18S**			**20S is made, exported and converted into 18S**	
Sub-Group	a	b	c	a	c	a	b	c	a	b
rRNA species accumulating after depletion	*35S/23S A0,A1,A2,D inhibited A3 Delay*	*35S/23S/22S A1,A2,D inhibited A0 Delay*	*35S/23S/+1-D A0,A1,A2, inhibited A3 delay D? can occur with delay*	*35S/23S/20S A0,A1,A2,A3 delay*	*35S/23S/21S A0,A1,A2,A3 delay*	*35S/23S A0,A1,A2,A3 delay D is inhibited*	*35S/23S/22S A0,A1,A2,A3 delay D is inhibited*	*35S/23S/21S A0,A1,A2,A3 delay D is inhibited*	*35S/23S A0,A1,A2,A3,D delay*	*35S/23S/22S A0,A1,A2,A3,D delay*
RPS	RPS13 RPS14 RPS16	RPS1 RPS6 RPS8 RPS9 RPS11 RPS23 RPS24	RPS27	RPS5	RPS18 RPS19	RPS15	RPS10 RPS26 RPS28	RPS0 RPS2 RPS3 *RPS20 **	RPS30	RPS7 RPS31

Figure 28: Summary of the effects of r-protein depletion on the 18S rRNA processing pathway.
* Note that 20S rRNA is exported almost with wildtype efficiency in rpS20-depleted cells, but cytoplasmic processing does not take place.

2. *In vivo* assembly pathway of SSU r-proteins.

As was shown in the first part of this work, depletion of single SSU r-protein inhibits different steps of the SSU rRNA maturation. In *E. coli*, r-proteins were shown to assemble *in vitro* according to a defined interdependent hierarchical pattern (see introduction 3. for details). Thus suggesting that depletion of a single r-protein could interfere with the assembly of other r-proteins.

Furthermore, it is not clear how ribosome assembly correlates with rRNA maturation since knowledge is lacking about the order, timing and compartmentalisation to assemble eukaryotic r-proteins *in vivo*.

To directly analyse the assembly states of the different eukaryotic small subunit precursor rRNAs the interaction of each r-protein of the yeast *S. cerevisiae* with pre-rRNA was systematically investigated. For this purpose, a collection of single tagged SSU r-proteins was generated in order to affinity-purify them and to determine their respective associated mature and pre-mature rRNA by Northern blotting.

Finally, assembly states of eukaryotic SSU rRNA precursors with SSU r-proteins were determined in mutant strains.

2.1. A collection of tagged SSU r-proteins to study *in vivo* assembly of SSU r-proteins.

The coding sequences of SSU r-proteins were cloned in fusion either with a N or C terminal Flag sequence and expressed under the control of (1) the conditionnal GAL1 promoter and (2) under the control of the RPS28 promoter to support constitutive expression. Constructs complementing growth in the genetic background of the complete knockout of the corresponding SSU r-protein genes were selected (see for details of construction materials & methods 6.).

Twenty-four out of 26 SSU r-proteins tested could be expressed from a multi-copy vector under the control of a constitutive promoter as either N- or C-terminal Flag-tagged proteins, which complemented the lethal phenotypes of the corresponding chromosomal gene deletions (see materials & methods table 1 and data not shown) demonstrating that these Flag-tagged alleles can fulfill their essential functions in ribosome biogenesis and translation. Flag-tagged alleles which were not able to complement the corresponding gene deletions were excluded from further analysis (e.g. Flag-rpS8, rpS8-Flag, Flag-rpS23, rpS23-Flag).

2.2. *In vivo* assembly of eukaryotic SSU r-proteins with pre-40S subunits.

After transformation of strain pGAL-RPS5 (ToY323) with the Flag-rpS vectors, single tagged SSU r-proteins were used to determine their respective associated mature and pre-mature rRNA by Northern blotting. These experiments showed that most of the tagged SSU r-proteins coprecipitated large amounts of mature 18S rRNA (Fig. 29 compare lanes 1 and 2) and 25S rRNA (not shown) in growing cells. This shows that the chosen Flag-fusion proteins are stably assembled into cytoplasmic, translationally active ribosomes, even in conditions of co-expression of the wildtype allele, although some of them lead to a slight delay of early rRNA processing (Flag-rpS11, Flag-rpS13, rpS26-Flag). Flag-fusion proteins which did not efficiently coprecipitate the mature rRNA species were excluded from further analysis, even if they complemented the loss of function of the corresponding gene disruptions (e.g. Flag-rpS18, rpS18-Flag, Flag-rpS7, Flag-rpS30, rpS30-Flag). The efficiency of 18S rRNA precipitation indicates the assembly state of the mature 40S subunit in regard to the different Flag-fusion proteins and serves in the following experiments as an internal control for individual coimmunoprecipitation reactions. Interestingly, all Flag-tagged r-proteins selected according to the criteria described above - except rpS26 (N- and C-terminally Flag-tagged) - coprecipitated also a significant amount (up to 15%) of 20S rRNA (see Fig. 29 compare lanes 1 and 2). The low ratio of efficiency of 20S pre-rRNA versus 18S rRNA coprecipitation with rpS26 suggests a rather late stable assembly of rpS26 into pre-ribosomes. The other r-proteins seem to become stably associated within 20S rRNA-containing nascent pre-ribosomes. In contrast, the amounts of coprecipitated early precursors like 35S rRNA (0.1%-1.5%) and 23S rRNA (0.1%-3%) were clearly reduced although they varied between individual r-proteins (see Fig. 29 compare lanes 1 and 2). Interestingly, the same immunoprecipitation pattern was observed using strains exclusively expressing the individual Flag-tagged RPS alleles (for summary of the quantifications of these experiments, see Fig. 35A) and in some exemplary strains expressing protein A tagged rpS (data not shown). In addition, no significant rRNA degradation was observed in the course of these experiments (see Fig. 30A) Furthermore, work of the Baserga laboratory (Bernstein et al., 2004) showed very similar characteristics for HA-tagged r-proteins rpS4 and rpS6 in regard to their interaction with early and later precursor rRNA. Altogether, these experiments minimise the possibility that these results can be explained by general accessibility problems of the tags in early pre-ribosomes for all of the rpS alleles tested. Finally, Flag-tagged Noc4p and Nop14p coimmunoprecipitated very efficiently early precursor rRNA, showing that interactions with

early pre-ribosomes are in principal well detectable using this methodology (Fig. 29, upper left panel and data not shown).

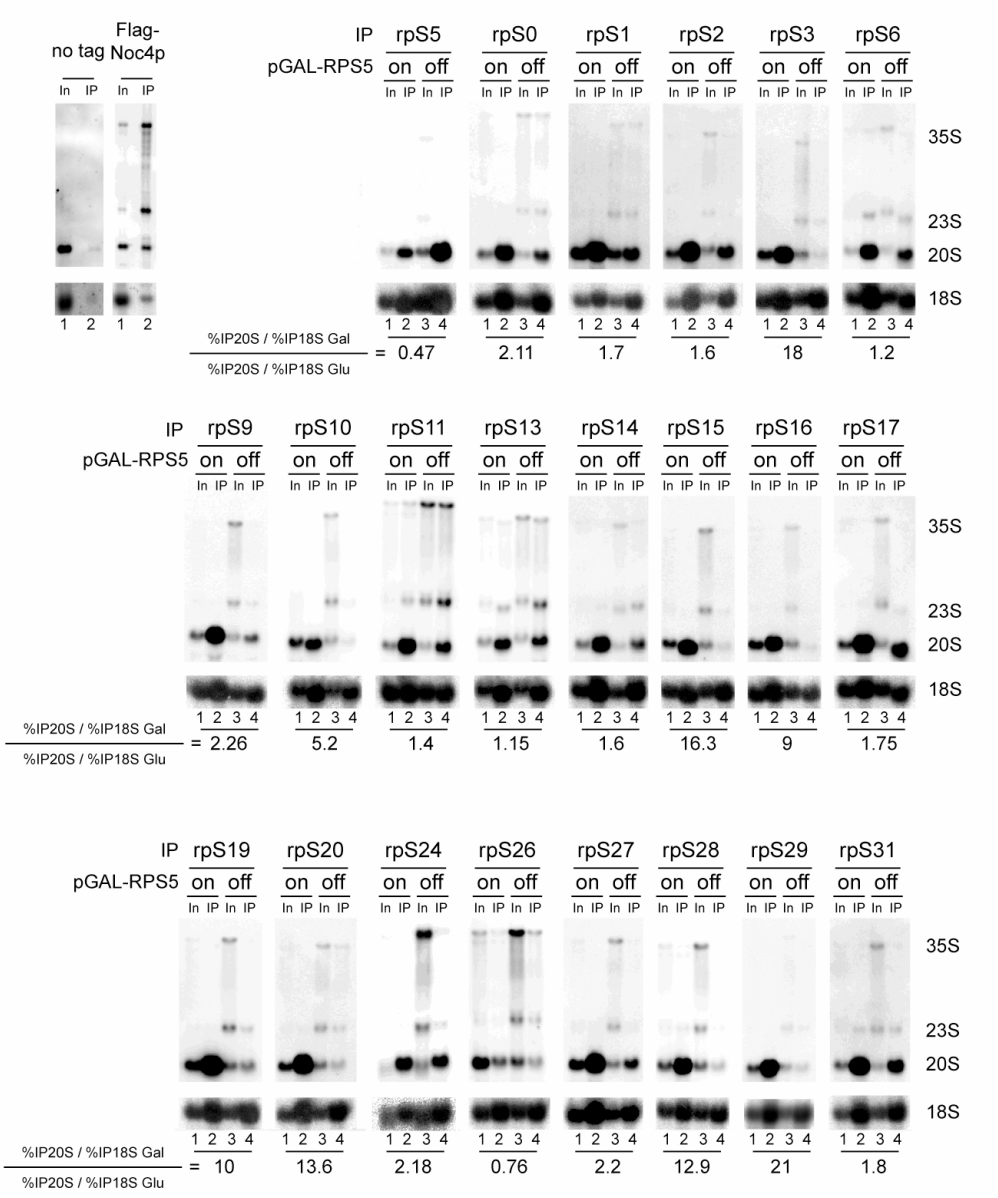


Figure 29: Analysis of r-protein association with (precursor) SSUs before and after *in vivo* depletion of rpS5.

Yeast strain (ToY323), expressing RPS5 under the control of a galactose inducible promoter was transformed with vectors supporting the constitutive expression of Flag-tagged SSU r-proteins. Positive transformants were selected and logarithmically growing cells were diluted to an OD of 0.3-0.5 and incubated 4 h in YP-galactose-containing media. Cultures were split; one half was further incubated 2 h in YP-galactose containing media (on) and the other half incubated 2 h in YP-glucose-containing media to shutdown the expression of RPS5 (off). Cell extracts were prepared and association of SSU Flag-r-proteins with their rRNA targets were analysed by RNA coimmunoprecipitation experiments (IP) followed by Northern blotting (see materials & methods 4.4. and 2.2.6.). Ribosomal RNA species were determined using probes complementary to the ITS1 (D-A2) and the 18S region (see materials & methods Table 4). Signals in input lanes (In) correspond to 1% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes).

A representative rRNA analysis of supernatants is shown in Fig. 30A.

In the upper left corner a corresponding analysis of a Flag-Noc4 and a no-tag-carrying strain is depicted.

Legend Figure 29 (continued): Signals of coimmunoprecipitated 20S rRNA and 18S rRNA were quantified as described in the materials & methods section. Percentages of coimmunoprecipitated (pre-) rRNA were determined. Efficiency of 18S rRNA precipitation serves as an internal control of the IP experiments and of the general assembly characteristics of the Flag-rpS fusion proteins. The efficiency of coimmunoprecipitated 20S rRNA in permissive condition ([%IP 20S rRNA / %IP 18S rRNA] in galactose) versus the efficiency of coimmunoprecipitated 20S rRNA in nonpermissive condition ([%IP 20S rRNA / %IP 18S rRNA] in glucose) were compared and expressed as the following ratio: [(%IP 20S rRNA / %IP 18S rRNA) in galactose / (%IP 20S rRNA / %IP 18S rRNA) in glucose]. A representative experiment from three independent experiments is shown.

However, it is still possible in the case of SSU r-protein precipitation that the large amount of mature ribosomes coprecipitated inhibits the efficient precipitation of earlier precursor. To exclude this possibility, competition experiments mixing equal protein amounts from whole cell extracts of strains expressing flag tagged allele of Noc4p and rpS13 were performed. As shown in Fig. 30B efficient precipitation of both early precursors and mature ribosomes is still possible in this condition, suggesting that the precipitation of large amount of mature ribosomes does not significantly influence the precipitation efficiency of the earliest pre-rRNA.

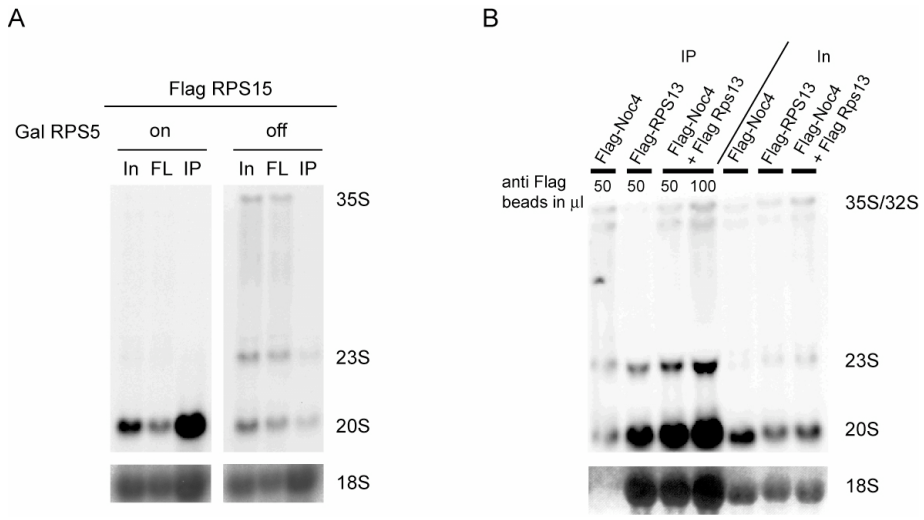


Figure 30: Control experiments.

(A) Immunoprecipitations of Flag-tagged rpS15 in yeast strain (ToY323), expressing RPS5 under the control of a galactose inducible promoter in permissive and non-permissive conditions were performed as described in Figure 29. Signals in input lanes (In) correspond to 1% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes). Signal in flow through lanes (FL) correspond to 1% of the supernantants (non bound material) after 90 min incubation at 4°C with anti-Flag agarose beads. A representative result is shown.

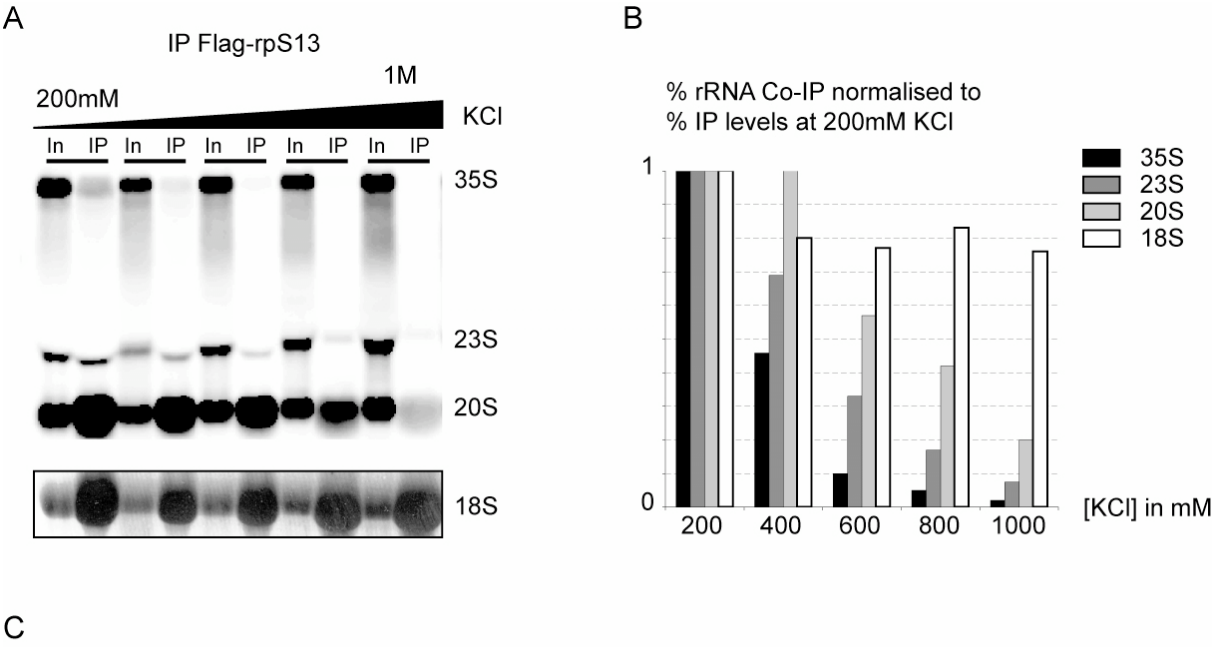
(B) Competition experiments mixing equal protein amount from whole cell extracts of strains expressing flag–tagged allele of Noc4 and rpS13 were performed.

Five mg of proteins whole cell extracts from strains Flag-Noc4 and untagged wildtype (Flag-Noc4 lane), or Flag–rpS13 and untagged wildtype (Flag-RPS13 lane), or Flag-Noc4 and Flag-rpS13 (Flag-Noc4 + Flag-RPS13 lane) were mixed and incubated with the indicated amount of anti-Flag agarose beads (IP) and immunoprecipitations were performed as described in Figure 29. Signals in input lanes (In) correspond to 1% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes).

In conclusion, most SSU r-proteins show a lower interaction affinity with early precursors suggesting that association of SSU r-proteins with the SSU precursor is somehow increased in the course of ribosome assembly. Alternatively early precursor r-protein composition could be more heterogeneous than for late precursor, thus leading to inefficient coimmunoprecitation of early pre-rRNA (or both possibilities).

In order, to determine whether r-proteins are associated with pre-rRNAs with different strength, coimmunoprecipitation experiments for Flag-rpS13, rpS14, and rpS20 using increasing salt conditions were performed (Fig. 31). Whereas association with early precursor (35S) rRNA was lost upon treatment with 600 mM KCl, interaction with 20S rRNA was still seen at concentrations of 600 mM KCl and 800 mM KCl, respectively. These experiments suggest that there is a salt labile but detectable association of many r-proteins with the earliest rRNA precursors. In fact, a few r-proteins have been documented in Miller spreads to associate co-transcriptionally with the nascent transcript (Chooi and Leiby, 1981). These data now demonstrate that at least some of the SSU r-protein interactions with early precursor (35S) rRNA are of functional relevance, since a large group of r-proteins supports cleavage events in the early rRNA precursors (see Fig. 28). These early processing steps are tightly linked to a structural rearrangement – presumably by rRNA folding and protein-protein interactions – through which the association of most r-proteins with 20S pre-rRNA is stabilised (see also summary Fig. 35A). This is in agreement with EM analysis which indicates a compaction of the rRNA/ r-protein complex during the *in vitro* assembly process of prokaryotic SSU (Mandiyan et al., 1991). Interestingly, the stability of interaction of early SSU-processing factors with precursor rRNA shows an inverse behaviour. SSU-processome components, like Flag-tagged Noc4p associate much stronger with early precursor (35S) rRNA than with the late precursor (20S) rRNA (see Fig. 29, upper left panel and Gallagher et al., 2004). This suggests that the co-transcriptionally established SSU-processome – rRNA complex (Osheim et al., 2004) undergoes a significant structural reorganisation upon early processing events and concomitant stable association of r-proteins which leads finally to the dissociation of the SSU-processome. In all cases examined, the stability of the interaction between r-proteins and 20S rRNA-containing particles reached comparable (Fig. 29, lanes 1, 2) but not identical levels to that of the same r-proteins with mature 18S rRNA (Fig. 31, compare immunoprecipitation of 18S and 20S rRNA at 1M KCl). This suggests that late (cytoplasmic) maturation events precede a further structural reorganisation of parts of the SSU

which could lead to the final mature SSU in agreement with recent work (Schafer et al., 2006).



C

Bait	rRNA co-precipitated	[KCl] in mM 200	400	600	800	1000
Flag-rpS13	35S	1	0,46	0,1	0,05	0,02
	23S	1	0,69	0,33	0,17	0,074
	20S	1	1	0,57	0,42	0,2
	18S	constant				

Bait	rRNA co-precipitated	[KCl] in mM 200	400	600	800	1000
Flag-rpS14	35S	1	0,25	0,07	ND	ND
	23S	1	0,4	0,15	ND	ND
	20S	1	0,52	0,32	ND	ND
	18S	constant				

Bait	rRNA co-precipitated	[KCl] in mM 200	400	600	800	1000
Flag-rpS20	35S	1	0,29	0,04	Bckg	Bckg
	23S	1	0,21	0,05	Bckg	Bckg
	20S	1	0,68	0,25	0,15	0,1
	18S	constant				

Figure 31: Influence of salt concentrations on the association of Flag-rpS13, Flag-rpS14 and Flag–rpS20 with (precursor) rRNA.

(A) Yeast strain expressing an N-terminal Flag-tagged version of the primary binder homologue rpS13 (ToY272) was grown in YP-galactose to an OD of 0.8-1. Association of Flag-rpS13 with (precursor)-rRNA was analysed as described in Fig. 29 except that the salt concentrations indicated (0.2, 0.4, 0.6, 0.8 and 1 M KCl) were present during cell breakage and coimmunoprecipitation procedures. Signal in input lanes (In) correspond to 1% of loaded cell extract onto the anti-Flag agarose beads (IP lanes).

(B) Signals of coimmunoprecipitated rRNA species from blot shown in (A) were quantified as described in the materials & methods section. Percentages of coimmunoprecipitated rRNA species were normalised to the percentage of coimmunoprecipitated rRNA determined at 200 mM KCl.

(C) Yeast strains expressing N-terminal Flag-tagged version of rpS13, rpS14, rpS20 homologue of *E. coli* primary binder S15 and tertiary binders S11 and S10 respectively were grown in YP-galactose to an OD of 0.8-1. Association of Flag-rpS13, Flag-rpS14 and Flag-rpS20 with (precursor)-rRNA were analysed as described in Fig. 29. Signals of coimmunoprecipitated rRNA species from the blots were quantified as described above. Percentage of coimmunoprecipitated rRNA species were normalised to the percentage of coimmunoprecipitated rRNA determined at 200 mM KCl.

In summary, (1) most SSU r-proteins interact weakly with the earliest rRNA precursor, (2) these weak interactions are - for some r-proteins - of functional relevance, and (3) SSU r-proteins interactions with pre-rRNA are stabilised in the course of the SSU maturation/ assembly. However, it is not clear how rRNA maturation events influence the stability of SSU r-proteins interactions with pre-rRNA.

2.3. *In vivo* assembly of eukaryotic SSU r-proteins with rRNA precursors after depletion of rpS5.

From the results described above, it is not clear which prerequisites exist for the *in vivo* formation of stable assembly intermediates of eukaryotic SSU r-proteins with the SSU precursor rRNA.

To this end, the interaction of SSU r-proteins and pre-rRNA after shutting off the expression of the SSU r-protein rpS5 was analysed.

First, *in vivo* depletion of rpS5 leads to a delay in early processing events and thereby to an accumulation of early (35S, 23S) rRNA precursors (see results 1. and summary Fig. 28). Accordingly, by increasing the half-life of these rRNAs, one can test, whether interactions with early precursors can be strengthened.

Second, in rpS5 depleted cells, 20S rRNA containing pre-ribosomes are not able to translocate from the nucleus to the cytoplasm (see results 1. and summary Fig. 28) as it happens under normal growth conditions (see introduction 2.2.2.3.). Therefore, this phenotype allows to examine how *in vivo* compartmentalisation of ribosome biogenesis in eukaryotes influences assembly of r-proteins with the nascent SSU.

Third, the eubacterial r-protein S7, homolog of rpS5, was shown to be an *in vitro* primary binder of bacterial mature 16S rRNA and is crucial for assembly of the SSU head structure (Mizushima and Nomura, 1970; Brodersen et al., 2002; Nierhaus, 2004; Talkington et al., 2005). Thus, the analysis of the assembly status of SSU precursors from rpS5 depleted cells will give insights in how both, the eukaryotic and the *in vivo* contexts influence the hierarchy of r-protein assembly.

The results of the experiment shown in Fig. 29 (compare lanes 1/2 and 3/4) indicate, that expansion of the half-life of early rRNA precursors by *in vivo* depletion of rpS5 is not sufficient to improve the apparent stability of their interaction with r-proteins (compare precipitation of 35S rRNA in lanes 2 and 4). Similar observations were made using exemplary r-proteins as baits (data not shown) after reducing rRNA processing kinetics, by incubating yeast cells at low temperature (Trapman and Planta, 1975).

Strikingly, after shutting down expression of rpS5, even interaction of a subgroup of SSU r-proteins with pre-ribosomes containing the late (20S) precursor rRNA is clearly weakened (Fig. 29; rpS3, rpS10, rpS15, rpS16, rpS19, rpS20, rpS28, rpS29 compare lanes 1/2 with lanes 3/4, see Fig. 35B, left panel, for a summary of the quantitation of these experiments)

demonstrating that these r-proteins require rpS5 for stable incorporation into the SSU precursor. This is especially interesting, since, if existing, the corresponding homologous counterparts in eubacteria belong to the group of r-proteins which localise to the head structure of the SSU (Wimberly et al., 2000; Spahn et al., 2001; Yusupov et al., 2001, see also Fig. 32A). Thus, these results show that (1) early processing events - which are delayed, but still occur in rpS5-depleted cells - are tightly linked to formation of a stable assembly intermediate of the body, but not of the SSU head structure. (2) Formation of a stable assembly intermediate of the SSU head structure strictly depends on the presence of rpS5 (and is not required for early processing events). Since *in vitro* formation of the eubacterial SSU head structure depends on the rpS5 homologue S7 (Mizushima and Nomura, 1970; Nomura and Erdmann, 1970; Nierhaus, 2004; Talkington et al., 2005), these results suggest a remarkable analogy (Bubunenko et al., 2006) between general principles of the *in vivo* and *in vitro* r-protein assembly pathways of eukaryotic and prokaryotic ribosomes. Apparently, the enormous increase in number of essential accessory factors and structural components introduced in the *in vivo* context of eukaryotic ribosome biogenesis does not change the main aspects of the general hierarchy of r-protein assembly.

Surprisingly, rpS0, the homologue of eubacterial S2, does not depend on rpS5 in its stable interaction with precursor rRNA, as it would be expected by the eubacterial *in vitro* assembly map (Mizushima and Nomura, 1970; Nomura and Erdmann, 1970; Nierhaus, 2004; Talkington et al., 2005). A comparison of the (predicted) 3D structure of eubacterial S2 and eukaryotic rpS0 in the SSU indicates that this difference in assembly dependence correlates with the reduced contact interface between the eukaryotic rpS0 and the SSU head structure (compare Fig. 32B to Fig. 32C).

In summary, it can be concluded that (1) early processing events - which are delayed, but still occur in rpS5-depleted cells - are tightly linked to formation of a stable assembly intermediate of the body, but not of the SSU head structure. (2) Formation of a stable assembly intermediate of the SSU head structure strictly depends on the presence of rpS5 (and is not required for early processing events). (3) R-protein assembly pathways in eukaryotes *in vivo* display striking similarities to the one shown *in vitro* for prokaryotic ribosomes. (4) Finally, previous data demonstrated that rpS5 is strictly required for nuclear export of the SSU-precursor (see results 1. and summary Fig. 28). Thus, the data shown in Fig. 29 indicate that the formation of a stable assembly intermediate of the SSU body in the absence of rpS5

occurs in the nucleus but is not sufficient to enable a consecutive translocation through the nuclear pores.

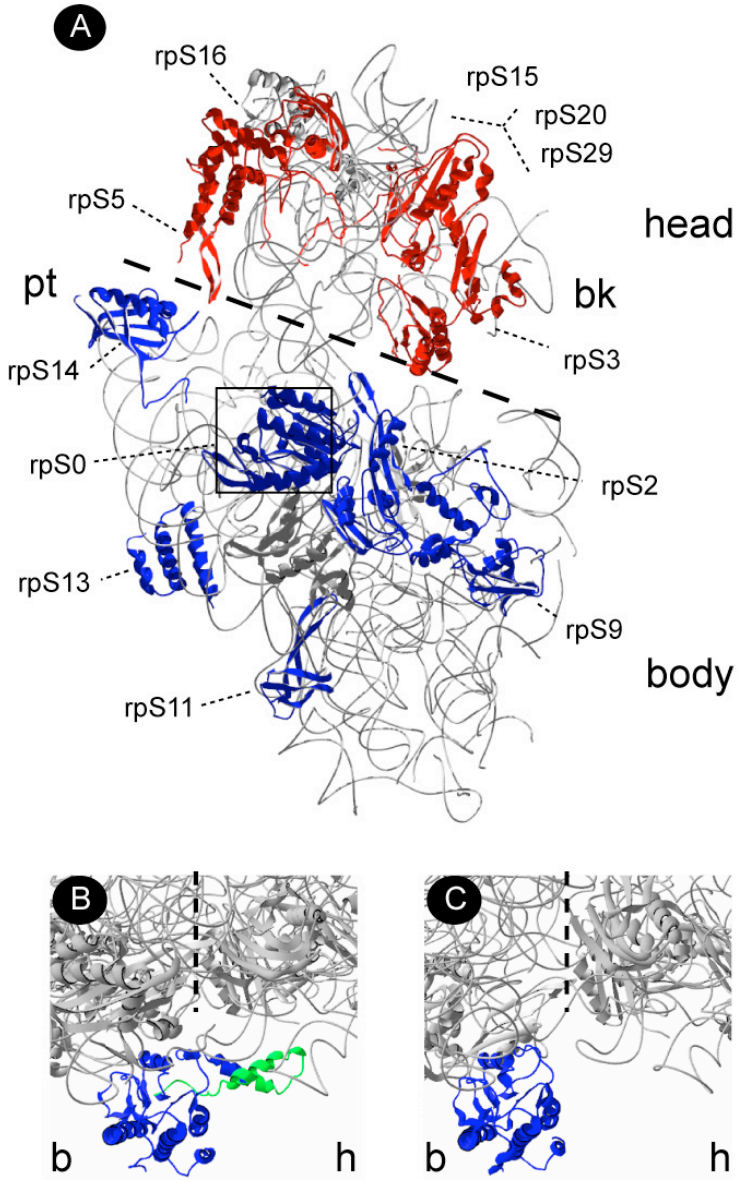


Figure 32: Structural view of the eukaryotic small ribosomal subunit.

Structural landmarks are indicated as following: b for body; h for head; pt for platform; bk for beak. The thick dashed line indicates the "border" between the body and the head structure.

(A) Structure of the eukaryotic SSU.

Eukaryotic SSU structure at 11.5 Å according to pdb file 1S1H (Spahn et al., 2004) shown from the solvent side. R-proteins analysed in this study which were localised and modeled by Spahn et al. (Spahn et al., 2004) according to their homology with prokaryotic counterparts are coloured as follows; red, r-proteins associated with the head structure; blue r-proteins associated with the body structure of the SSU. The 18S rRNA and r-proteins modeled but not analysed in this study are indicated in grey. The square indicates rpS0.

(B) Structure of the prokaryotic rpS0 homologue S2 and its rRNA/ r-protein neighborhood in the *T. thermophilus* SSU at 5.5 Å according to pdb file 1J5E (Wimberly et al., 2000). S2 is coloured in blue, the central protein domain of 46 amino acids of S2 which is not conserved in the eukaryotic counterpart rpS0 is coloured in green.

(C) Structure of rpS0 and its rRNA neighborhood in *S. cerevisiae* according to pdb file 1S1H (Spahn et al., 2004). rpS0 is coloured in blue, 18S rRNA and other r-proteins are in grey.

2.4. Assembly of eukaryotic SSU r-proteins with 20S pre-rRNA after *in vivo* depletion of rpS15.

To further compare the principles of eukaryotic SSU *in vivo* assembly with the hierarchical prokaryotic *in vitro* assembly map the assembly status of the precursor SSU after *in vivo* depletion of rpS15 was analysed. S19, the prokaryotic homologue of rpS15, was described to act as secondary binder in the *in vitro* assembly map of the prokaryotic SSU head domain, downstream of the primary binder S7, the prokaryotic homologue of rpS5 (Mizushima and Nomura, 1970; Nomura and Erdmann, 1970; Nierhaus, 2004; Talkington et al., 2005). The data shown in Fig. 33 indicate that *in vivo* depletion of rpS15 specifically affects the assembly of many r-proteins (rpS5, rpS10, rpS16, rpS20, rpS28, rpS29) into the head-like structure, not the body (rpS13 and rpS14) of the SSU. Interestingly, quantification of the results (summarised in Fig. 35B) shows that *in vivo* depletion of the primary binder-homologue rpS5 has a much stronger impact on formation of the head-like structure than depletion of the secondary binder-homologue rpS15. Consistently, absence of the primary binder-homologue rpS5 affects assembly of the secondary binder homologue rpS15 much more drastically than *vice versa*.

These results strengthen the assumption that general principles of the prokaryotic *in vitro* assembly pathway apply also for assembly of ribosomes in eukaryotic cells. Further analysis will be needed to show exactly how both the eukaryotic and the *in vivo* context influences assembly of r-proteins. In addition, the 15 SSU r-proteins which have no homologous counterparts in eubacteria have to be positioned in an "*in vivo* assembly map".

Furthermore, the analysis of the SSU assembly status in the rpS15 mutant reveals an additional *in vivo* assembly intermediate of the eukaryotic SSU head. The corresponding phenotypes in ribosome biogenesis differ in several aspects from those observed after depletion of rpS5: no apparent delay of early rRNA processing events, a strongly delayed nuclear export of 20S rRNA and a strong inhibition of late cytoplasmic rRNA processing events (see results 1. and summary Fig. 28). Apparently, ongoing formation of a stably assembled head-like structure has a strong impact in the ability of the SSU to leave the nucleus.

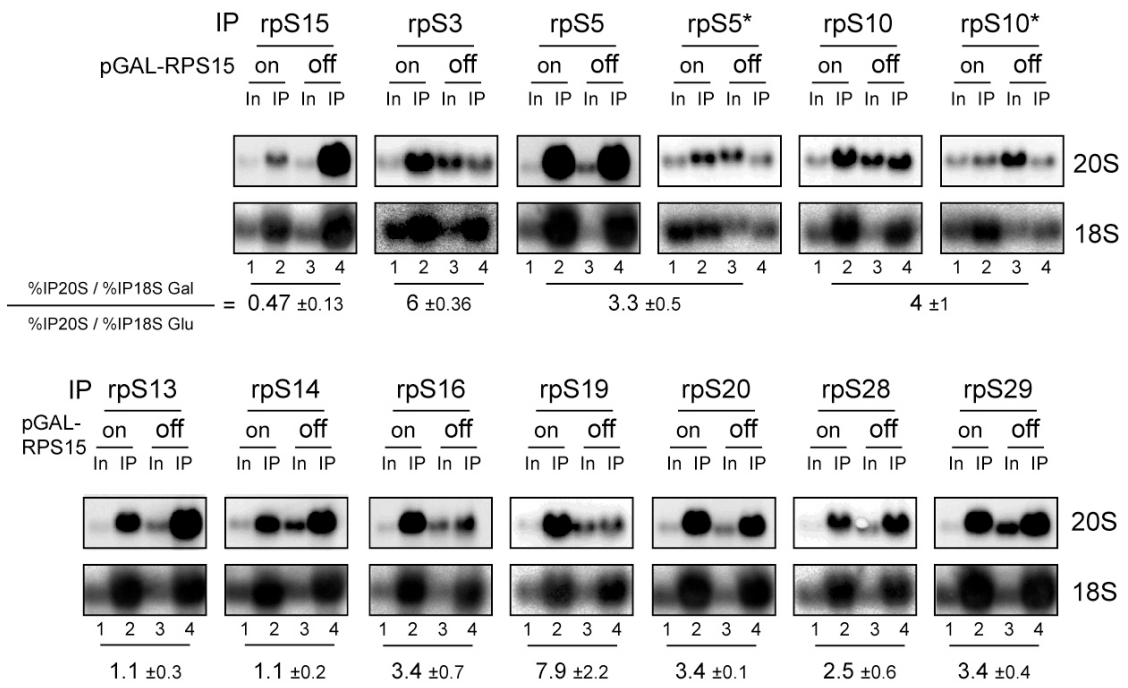


Figure 33: Analysis of r-protein association with (precursor-) SSUs before and after *in vivo* depletion of rpS15.

Yeast strain (ToY89), expressing RPS15 under the control of a galactose inducible promoter was transformed with vectors supporting the constitutive expression of Flag-tagged SSU r-proteins.

RNA coimmunoprecipitation experiments were performed as described in Fig. 29.

Signals in input lanes (In) correspond to 1% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes). For rpS5* and rpS10* signals in input lanes (In) correspond to 3% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes).

Signals of coimmunoprecipitated 20S rRNA and 18S rRNA were quantified as described in the materials & methods section. Quantifications were performed using two different loadings as exemplified for rpS5/ rpS5* and rpS10/ rpS10* (upper panel).

Percentages of coimmunoprecipitated rRNA were determined. Efficiency of 18S rRNA precipitation serves as an internal control of the IP experiments and of the general assembly characteristics of the Flag-rpS fusion proteins. The efficiency of coimmunoprecipitated 20S rRNA in permissive condition ([%IP 20S rRNA / %IP 18S rRNA] in galactose) versus the efficiency of coimmunoprecipitated 20S rRNA in non-permissive condition ([%IP 20S rRNA / %IP 18S rRNA] in glucose) were compared and expressed as the following ratio: [(%IP 20S rRNA / %IP 18S rRNA) in galactose / (%IP 20S rRNA / %IP 18S rRNA) in glucose]. Ratios are indicated as an average of the values determined from two independent experiments and two different loadings for each experiments.

2.5. *In vivo* assembly status of nascent nuclear SSU after inactivation of the ribosome exportin Crm1p.

As shown earlier depletion analysis demonstrated that rpS5 blocks and rpS15 strongly affects nuclear export of the SSU-precursors (results 1. and summary Fig. 28). In addition, rpS5 and rpS15 depletion is weakening the interaction with (20S) rRNA precursors of an r-protein group forming a head-like structure. Moreover, individual depletion of these r-proteins impairs (efficient) translocation of pre-ribosomes through the nuclear pore (results 1. and

summary Fig. 28). Finally, the data shown in Fig. 29 indicate that formation of a stable assembly intermediate of the SSU body in the absence of rpS5 occurs in the nucleus but is not sufficient to enable a consecutive translocation through the nuclear pores.

Accordingly, one can ask whether formation of a stabilised SSU head-like structure precedes nuclear export.

Therefore, association of SSU r-proteins with 20S rRNA precursor was analysed when nuclear export was impaired through inactivation of the ribosomal exportin Crm1p (Moy and Silver, 1999; Leger-Silvestre et al., 2004).

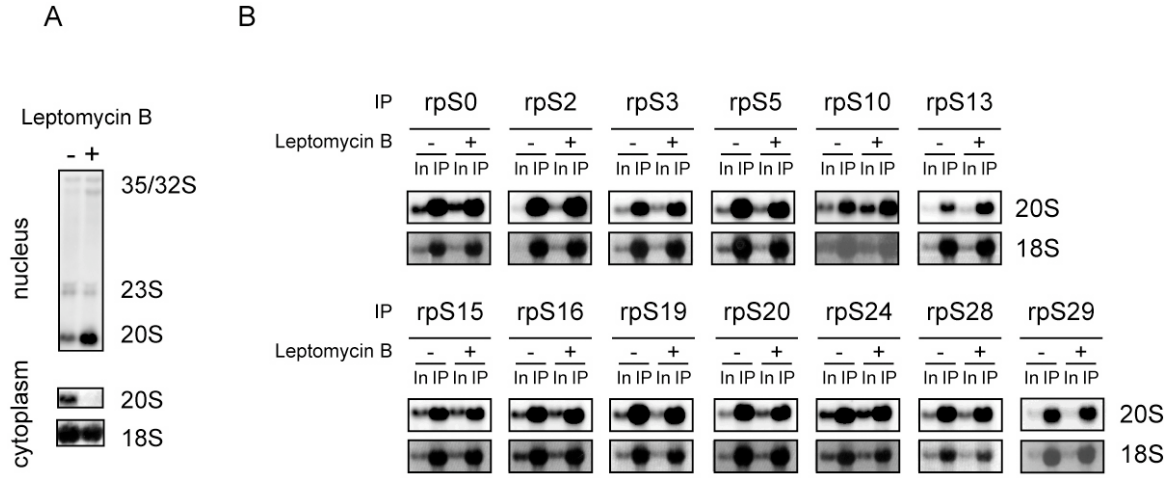


Figure 34: Association of SSU r-proteins with (precursor-) SSUs after inactivation of exportin Crm1p.

(A) Northern analysis of SSU (precursor)-rRNA of nuclear/ cytoplasmic fractions of cells treated with (+) or without (-) 100 nM leptomycin B (LMB) (see materials & methods).

To control the localisation of 20S rRNA before and after inhibition of the exportin Crm1p with LMB, nuclear and cytoplasmic fractions of a yeast strain (ToY376) carrying a leptomycin B sensitive allele of exportin Crm1 (Neville and Rosbash, 1999) were prepared after treatment with (+) or without (-) 100 nM leptomycin B for 45 minutes as described in (Leger-Silvestre et al., 2004). A representative experiment is shown.

(B) Association of Flag-tagged SSU r-proteins with (precursor-)-SSUs after inactivation of exportin Crm1p.

Yeast strain (ToY376) carrying a leptomycin B sensitive allele of exportin Crm1 (Neville and Rosbash, 1999) was transformed with vectors supporting constitutive expression of Flag-tagged SSU r-proteins. Logarithmically growing transformants were diluted to an OD of 0.3-0.5 and incubated 2 h in YP-glucose. Cultures were split and further incubated for 45 min with (+) or without (-) 100 nM of leptomycin B. Cell extracts were prepared and association of SSU Flag-r-proteins with their rRNA targets was analysed by RNA coimmunoprecipitation experiments followed by Northern blotting (see materials & methods). Ribosomal RNA species were determined using probes complementary to the ITS1 (D-A2) and the 18S region. Signals in input lanes (In) correspond to 1% of loaded cell extracts onto the anti-Flag agarose beads (IP lanes).

As demonstrated previoulsly (Moy and Silver, 1999; Leger-Silvestre et al., 2004 and in Fig. 34A), Crm1p inhibition results in an almost complete block of 20S rRNA precursor export to the cytoplasm. In contrast to rpS5 and rpS15 depletion, inhibition of Crm1p dependent SSU export does not prevent stable association of all SSU r-proteins that form a head-like, rpS5–dependent structure (Fig. 34B see rpS3, rpS10, rpS15, rpS16, rpS19, rpS20, and rpS28). These results demonstrate that it is further possible to dissect ribosomal assembly and nuclear export in several crucial steps. Different assembly states can be defined, and related to different effects on nuclear export (Fig. 35B). These data also indicate, that the establishment of a stably assembled SSU head-like structure within the nucleus represents a necessary prerequisite to allow efficient Crm1-mediated passage through the nuclear pores (see summary in Fig. 35B).

2.6. Summary on the *in vivo* assembly pathway of SSU r-proteins.

In summary, the results shown in the second part of this work suggest (1) that most SSU r-proteins interact weakly with the earliest rRNA precursors (2) these weak interactions are - for some r-proteins - of functional relevance. (3) SSU r-protein interactions with pre-rRNA are stabilised in the course of the SSU assembly. (4) This stabilisation apparently correlates with rRNA maturation events. (5) Formation of a stable assembly intermediate of the body-like, but not of the SSU head-like structure is tightly linked to early processing events (A0, A1, and A2 cleavages). (6) Formation of a stable assembly intermediate of the SSU head-like structure depends on the presence of rpS5 (and is not strictly required for early processing events). (7) Key aspects of the *in vivo* assembly of eukaryotic r-proteins into distinct structural parts of the SSU are similar to the *in vitro* assembly pathway of their prokaryotic counterparts (e.g. body-like formation independent from head-like formation, absence of primary binder-homologue affects assembly of the secondary binder-homologue much more drastically than *vice versa*). (8) Formation of a stable assembly intermediate of the SSU body occurs in the nucleus but is not sufficient to enable a consecutive translocation through the nuclear pores. (9) Formation of a stable assembly intermediate of a SSU head-like structure precedes and is required for (efficient) nuclear export. (10) Final cytoplasmic maturation events, which then lead to the mature SSU, require stable assembly of the SSU head-like structure and are accompanied with a further SSU reorganisation.

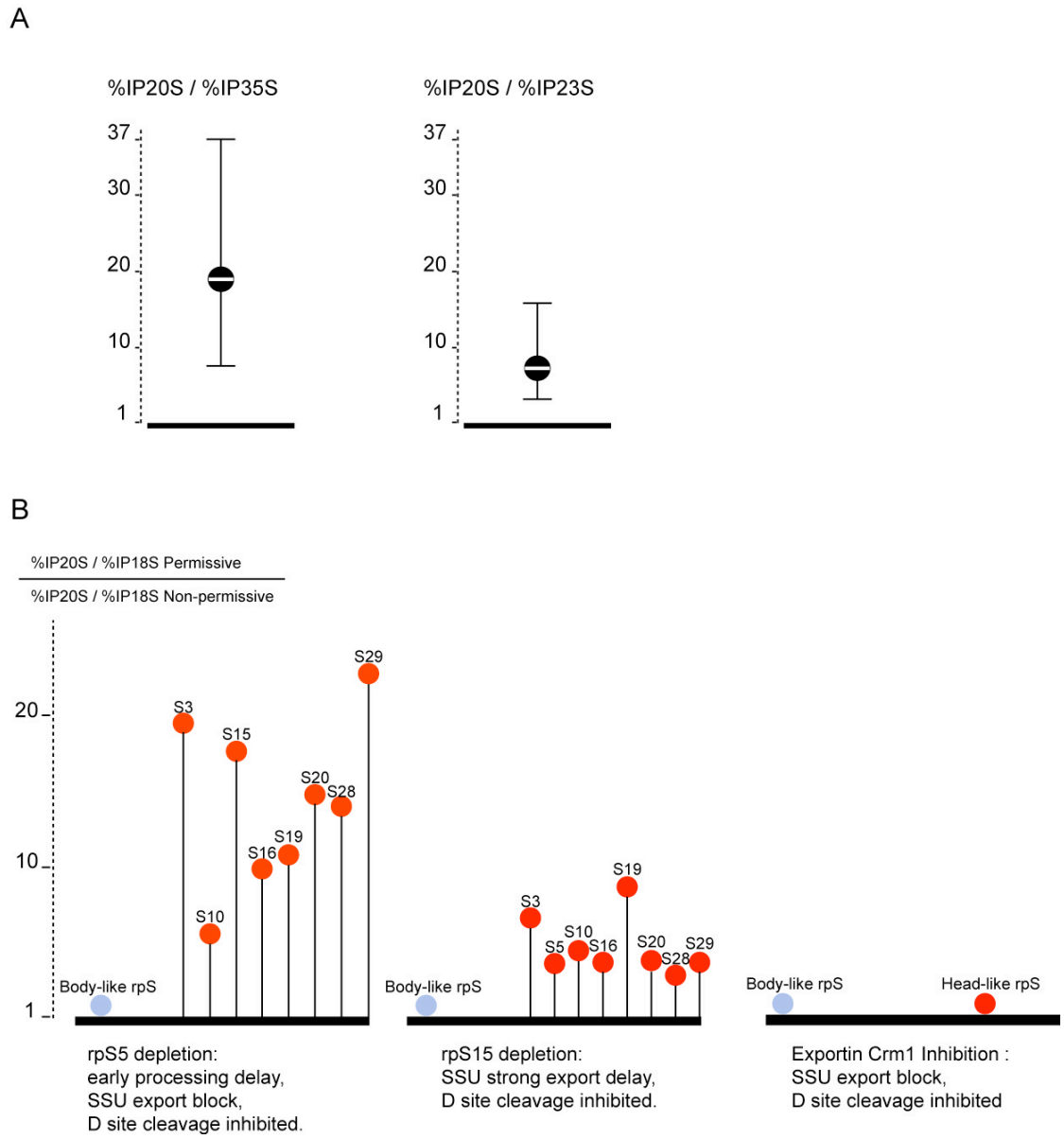


Figure 35: Summary of *in vivo* analysis of r-proteins association with precursor SSUs in conditional mutants in permissive and non-permissive conditions.

(A) Schematic representation of relative coimmunoprecipitation efficiency of 20S rRNA versus 35S rRNA (left panel) and 20S rRNA versus 23S rRNA (right panel) with Flag-tagged rpS.

The percentages of coimmunoprecipitated rRNA species were determined from strains carrying each one of 21 Flag-tagged rpS as the only source for this r-protein.

Ratio between percentage of coimmunoprecipitated 20S rRNA vs 35S rRNA (left panel) and ratio between percentage of coimmunoprecipitated 20S rRNA vs 23S rRNA (right panel) were determined. The Y-axis indicates how many times more efficient 20S rRNA was coprecipitated than 35S rRNA and 23S rRNA respectively. The minimum and maximum value for these ratios (parallel bar) were plotted on a graphic representation and the respective ratios average indicated with a white lane in a ball. Average [%IP 20S rRNA / %IP 35S rRNA] = 18.3± 8.3 and average [%IP 20S rRNA / %IP 23S rRNA] = 7.7± 3.3.

(B) Schematic representation of relative coimmunoprecipitation efficiency of 20S rRNA with Flag-tagged r-proteins in permissive versus non-permissive condition in mutants for *rpS5* (left panel), *rpS15* (center panel) and *crm1* (right panel) are shown. Quantified results from figures 29, 33 and 34 were plotted on a graphic representation according to the following formula: ([%IP 20S rRNA / %IP 18S rRNA] in permissive conditions) / ([%IP 20S rRNA / %IP 18S rRNA] in non-permissive conditions). The Y-axis indicates how many times more efficient 20S rRNA was coprecipitated in permissive versus non-permissive conditions. R-proteins supposed to assemble in the body-like part of the SSU are indicated in blue, in the head-like part in red.

3. Function of SSU r-proteins in translation.

3.1. Are some essential SSU r-proteins involved in protein translation?

SSU r-proteins rpS7, rpS30, and rpS31 are essential for cell viability, however after depletion of the respective SSU r-proteins mature 18S rRNA could still be significantly produced (see results 1. and summary Fig. 28). Since mature 18S rRNA can be engaged into translating ribosomes, it is possible that these SSU lacking one of these SSU r-proteins are impaired in translation function.

This possibility was investigated by analysing the formation of polysomes in strains in which the respective SSU r-proteins were depleted.

Depletion of the essential SSU r-proteins rpS30, rpS31, and rpS7 showed a significant reduction in free 40S subunits and polysomes, whereas the levels of 80S and/ or free 60S ribosomes were increased (see Fig. 36A). Possible explanations for this result is that 40S subunits are incorporated into 80S ribosomes that are either not associated with mRNA (Hartwell and McLaughlin, 1969; Cigan et al., 1991) or are incompetent for translation initiation or elongation. Accordingly, the polysomes/ 80S ratio drops (Deloche et al., 2004).

In contrast, mutant strains with a pronounced defect in 40S biosynthesis show a clear reduction of the 40S peak and, in addition, a decrease in both 80S and polysomes peaks (but not in free 60S subunits)(Fig. 36A, Gal RPS0, 2 h YPD incubation).

Consistent with a role of rpS30 in ribosome function, a single knockout of one of the two RPS30 alleles also resulted in growth inhibition in the presence of cycloheximide (Fig. 36B).

Cycloheximide is an inhibitor of protein biosynthesis (Dresios et al., 2001) which blocks the peptidyltransferase activity of mRNA-associated ribosomes, thereby inhibiting both translation initiation and elongation.

In addition, deletion of RPS30B confers paromomycin sensitivity (Fig. 36C). Paromomycin is an aminoglycoside antibiotic which binds to the A-site of the ribosome, and interferes with the loading of aminoacyl-tRNA, inducing amino acids misincorporation during protein synthesis.

Altogether, these data suggest a possible role in translation of the SSU r-proteins rpS30, rpS7, and rpS31 but it remains to be shown how these SSU r-proteins affect ribosome function.

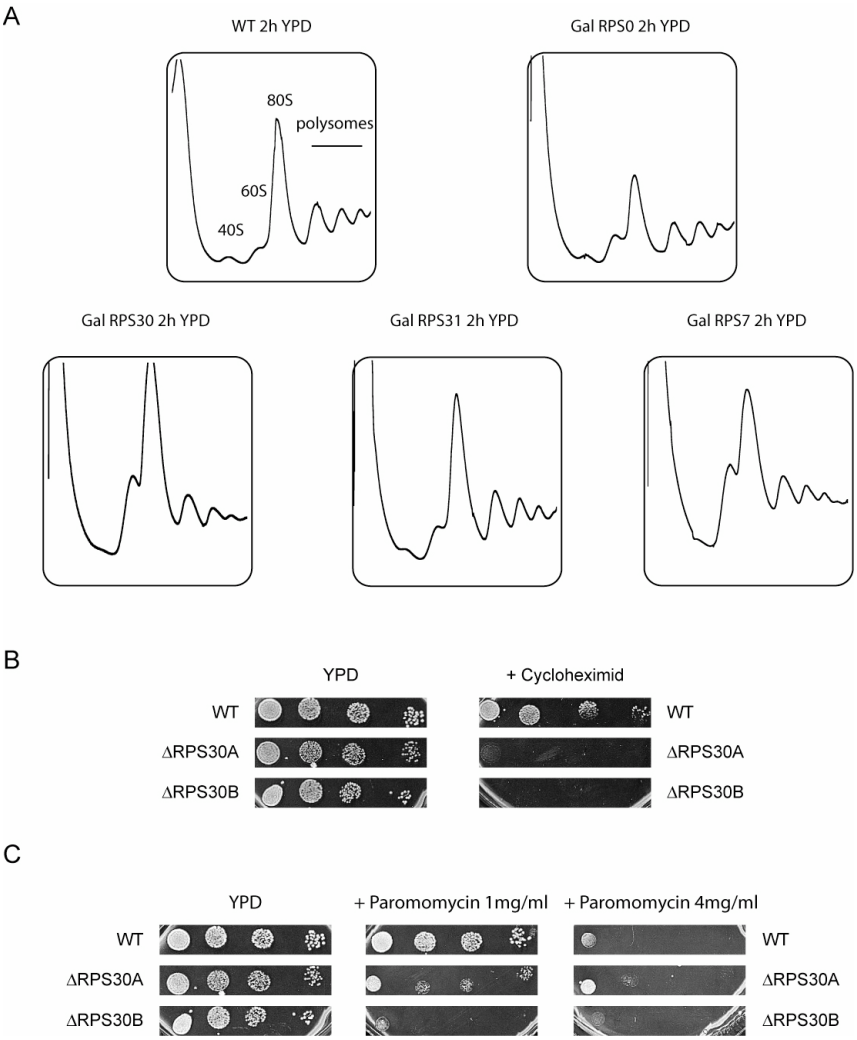


Figure 36: R-proteins mutants that allow production of mature 18S rRNA are impaired in ribosome function.
(A) Comparison between polysome gradients from strains deficient in 18S rRNA production (RPS0) and conditional lethal strains that still produce 18S rRNA (RPS30, RPS31, and RPS7). The strains were depleted in r-protein expression for 2 h in glucose-containing medium, treated with cycloheximide, and fractionated on an 8%–36% sucrose gradient.
(B) Cycloheximide sensitivity of strains with deletions of genes RPS30A or RPS30B. The dilution series were plated on YPD with and without 0.5 □g/ml cycloheximide.
(C) Paromomycin sensitivity of strains with deletions of genes RPS30A or RPS30B. The dilution series were plated on YPD with and without 1g/ml or 4g/ml paromomycin.

3.2. Are the known non-essential SSU r-proteins involved in protein translation?

The SSU r-proteins rpS12 and rpS25 are non-essential for cell viability in the condition tested. However, since these strains display slight growth defects it cannot be excluded that these SSU r-proteins play a role in modulation/ efficiency of protein synthesis.

Strains producing mature SSUs lacking the respective SSU r-protein rpS12 or rpS25 were analysed for their ability to assemble polysomes. Polysome profiles obtained from strains

lacking either rpS12 or rpS25 were indistinguishable from those of wildtype cells (Fig. 37), indicating that both proteins are not required for formation of mRNA-associated ribosomes.

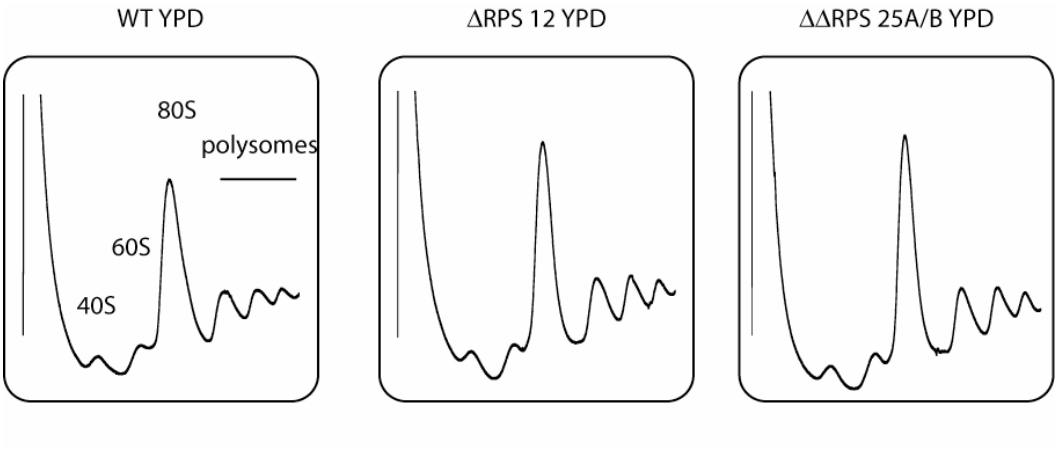


Figure 37: Absence of the non-essential r-proteins does not affect formation of polyribosomes.
Polysome gradients of strains depleted in the non-essential ribosomal proteins rpS12 and rpS25 (growth on YPD). Cycloheximide treated cell extracts were fractionated on a gradient containing 8%–36% sucrose. Absorbance at 260 nm was monitored.

Next, sensitivity against cycloheximide and paromomycin were analysed in strains lacking the respective protein.

In presence of 0.5 □g/ml cycloheximide, wildtype cells show only a slight growth reduction whereas strains lacking the SSU r-proteins rpS12 or rpS25 are significantly inhibited in growth (Fig. 38A).

Interestingly, in presence of 4 mg/ml of paromomycin growth of wildtype cells is clearly inhibited, while in strains lacking rpS12 growth is not affected. An intermediate phenotype is observed in strains lacking rpS25, in presence of 4 mg/ml of paromomycin, cell growth is inhibited however to a lesser extent than in wildtype strains (Fig. 38B).

These data show that the SSU r-proteins rpS12 and rpS25 are not required to form properly assembled polysomes, however sensitivity to inhibitors of translation of cells lacking these r–proteins strongly suggest a role of these SSU components in modulating ribosome function. In which specific step of protein translation these SSU r-proteins are involved remains to be determined.

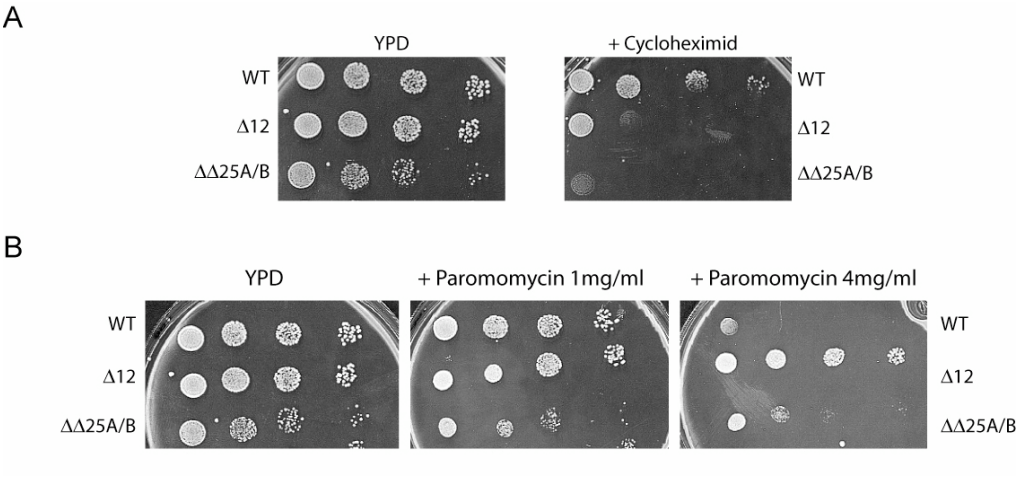


Figure 38: Absence of the non-essential r-proteins results in an altered sensitivity of yeast cells to translation inhibitors.

(A) Cycloheximide sensitivity of strains with deletions of genes RPS12 or RPS25A/B. The dilution series were plated on YPD with and without 0.5 g/ml cycloheximide.

(B) Paromomycin sensitivity of strains with deletions of genes RPS12 or RPS25A/B. The dilution series were plated on YPD with and without 1g/ml or 4g/ml paromomycin.

4. Relative stoichiometry of SSU r-proteins.

Previous works analysed the overall stoichiometry of individual SSU and LSU r-proteins (Kruiswijk and Planta, 1974; Kruiswijk et al., 1978c). However, some SSU r-proteins were not yet identified in these studies. Furthermore usage of different nomenclatures for the r–proteins troubles the interpretation of these results.

With the help of individual Flag-tagged SSU r-proteins a preliminary determination of the relative stoichiometry was performed. Same amount of whole cell extract of yeast strains bearing complete knockout for the SSU r-proteins complemented with the respective Flag-tagged SSU r-proteins under the control of the GAL1 promoter were analysed by SDS-UREA-PAGE. Transblotted proteins were identified using a polyclonal antibody directed against the chromosomal encoded SSU r-proteins rpS8 and a monoclonal antibody directed against the Flag tag.

Individual signals were quantified and stoichiometry is expressed as a ratio between the amount of rpS8 (used here as a normalisation reference) and the Flag-tagged SSU r-proteins.

The preliminary results shown in Fig. 39 suggest that most of the SSU r-proteins are present in similar amount in the SSU. Interestingly, Flag-tagged SSU r-proteins rpS30 and rpS31 are under represented in this study, indicating that only about 10% of the ribosomal SSUs contain one of these r-proteins.

In general, to be more conclusive these results need to be confirmed using a more accurate quantitative approach for example with the help of serial dilution and further statistical quantification. This is especially true for flag-tagged SSU r-proteins rpS18 or rpS3.

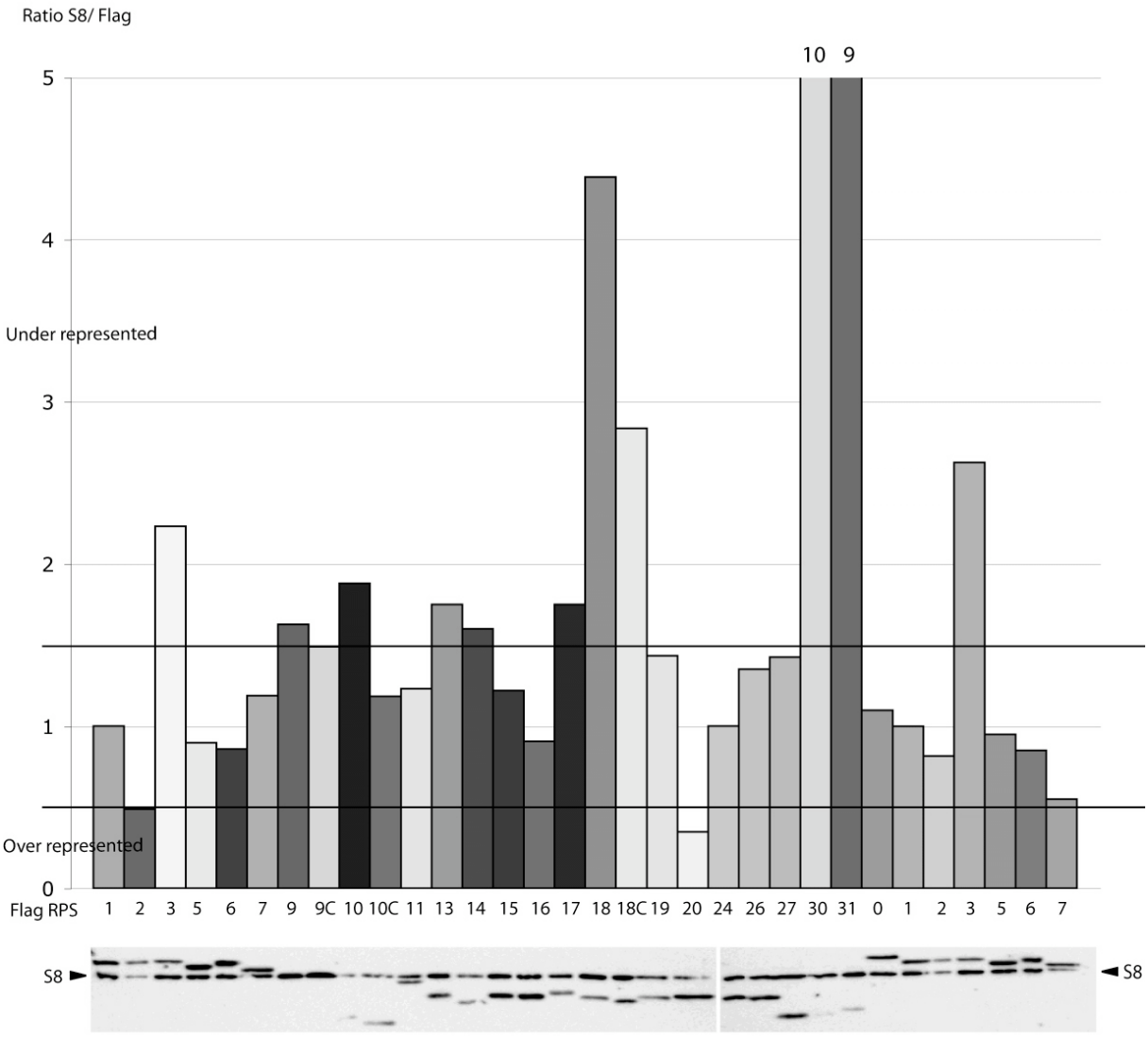


Figure 39: Relative stoichiometry of SSU r-proteins.

Equal amount of whole cells protein extracts as determined by Bradford assay were separated by SDS-Urea-PAGE. Transblotted proteins were detected by immunoblotting using anti-Flag and anti-rpS8 antibodies.
Signals were quantified and expressed as a ratio between rpS8 signals vs Flag-tagged-rpS signals.

DISCUSSION & PERSPECTIVES

This work describes the development and the usage of systematic approaches to study the roles of yeast SSU r-proteins in function, maturation, export and assembly of the SSU.

1. R-proteins and SSU biogenesis.

1.1. R-protein assembly and pre-18S rRNA maturation/ transport are interconnected.

1.1.1. Connection between r-protein assembly and pre-18S rRNA processing.

The results described above (results 1.) demonstrate that individual r-proteins control different pre-18S rRNA processing steps. In addition, the status of assembly of r-proteins is closely linked to rRNA processing events, suggesting that r-protein assembly and rRNA maturation events are functionally connected.

As shown in the results part, most of the tagged SSU r-proteins coprecipitated large amounts of mature 18S rRNA and 25S rRNA in growing cells confirming their incorporation into cytoplasmic, translationally active ribosomes. Interestingly, all r-proteins analysed - except rpS26 - coprecipitated also a significant amount of 20S rRNA, the immediate precursor of mature 18S rRNA suggesting that most r-proteins become stably associated within 20S rRNA-containing nascent pre-ribosomes. In contrast, the amounts of coprecipitated early precursors like 35S rRNA were clearly reduced although they varied between individual r-proteins. In addition, coimmunoprecipitation experiments using increasing salt conditions indicate that there is a salt labile but detectable association of many r-proteins even with the earliest rRNA precursors. Thus, suggesting that the interaction between the SSU r-proteins and their target rRNA are stabilised in the course of the *in vivo* SSU biogenesis.
Interestingly, eleven r-proteins are required for efficient early cleavages at A0, A1 and A2: after their depletion 35S and 23S rRNA accumulate, whereas 20S rRNA becomes not detectable. Thus, these results suggest that the weak interactions between the rpS and the early pre-rRNA described above are of functional relevance (see Fig. 28 and results section for details).

In rpS5-depleted cells early processing events (A0, A1 and A2) are delayed, but do still occur. Interestingly, a subset of r-proteins can assemble independently of rpS5, and form a body-like structure. Moreover many of these body-like r-proteins are required for early processing events, suggesting that these processing events are tightly linked to formation of a stable assembly intermediate of the body, but not of the SSU head structure.

Early processing events (A0, A1 and A2) require the SSU processome, a U3 snoRNA containing RNP. Interestingly, based on the observed interactions of U3 with the pre-rRNA, it was suggested that U3 snoRNA could stabilise a certain pre-rRNA conformation, bringing the 5' (ETS1) and 3' (ITS1) rRNA sequences into close contact inducing a circle-like structure of the pre-18S rRNA (see Hughes, 1996; Gerbi et al., 2003 and introduction 2.2.2.3. for details). Finally, most of the SSU processome components are released from the pre-18S rRNA after A0, A1 and A2 cleavages (see among others Fromont-Racine et al., 2003 and introduction 2.3. for details). These observations suggest that the SSU processome components could serve as a platform inducing a favourable conformational change of the pre-18S rRNA allowing a first (weak and functional) interaction between the rpS with the pre-rRNA. Apparently, immediately after or during release of the SSU processome from the transcript, a major structural rearrangement takes place, leading to a tight rRNA-r-protein interaction that is closely linked to cleavage at sites A0, A1, and A2. Thus, it is possible that some weak interactions of r-proteins with pre-rRNA are sufficient to induce structural changes either making the rRNA competent for cleavages, or allowing the recruitment of the nuclease(s) involved in these early processing steps. Alternatively cleavages at A0, A1, and A2 can serve as a signal for structural rearrangements, which then results in a tight interaction between r–proteins and 20S pre-rRNA.

In addition, the results described in this work suggest that after early processing at A0, A1 and A2, SSU nuclear export (see discussion & perspectives 1.1.2.), and cleavage at site D r–protein association with mature 18S rRNA gets further stabilised.

The analysis of the assembly status of the SSU after rpS15 depletion reveals an additional assembly intermediate of the eukaryotic SSU head-like structure. Interestingly rpS15 depleted cells show a strong inhibition of 20S rRNA processing at site D, suggesting that ongoing formation of a stable assembled head-like structure has a strong impact on the ability of the SSU to be properly matured in the cytoplasm.

Interestingly, many rpS components of the head-like structure are required for late cytoplasmic maturation. Therefore, it is possible that the final establishment of the head

structure, as a result of a tight association of r-proteins with rRNA is a prerequisite of D site cleavage or *vice-versa*.
In addition, recent work (Schafer et al., 2006) suggests that such SSU r-protein stabilisation can occur in the cytoplasm prior to the final rRNA maturation, which is in agreement with the observed difference of stability of the interaction between r-proteins and 20S rRNA and r-proteins and 18S rRNA containing particles.

Interestingly, the depletion of two r-proteins – rpS0 and rpS2 – shows inhibition of 20S rRNA processing at site D. However, these r-proteins do not assemble according to an rpS5 dependent head-like structure, and are not required for early processing events. Furthermore, another body-like r-protein - rpS14 – is found to participate directly or indirectly in D site cleavage (Jakovljevic et al., 2004). In addition, it was suggested that rpS14 interacts with a ribosome biogenesis factor – Fap7p – also required for D site cleavage (Granneman et al., 2005). Finally, all the r-proteins tested show a difference in their stability of interaction with the 20S and 18S rRNA, which is independent on their respective localisation in the mature SSU.
All together, these results suggest that D site cleavage is connected to further global rRNA conformational changes affecting r-protein stability in both the body- and head-like structure of the SSU.

It is possible that *in vivo* the body-like structure assembly status controls or facilitates formation of the head-like structure. On the other hand, the above r-proteins (e.g. rpS0, rpS2 and/ or rpS14) could serve as a platform to recruit factor(s) required for D site cleavage. However as shown in rpS15 depleted cells body-like r-protein assembly is not sufficient to trigger D site cleavage, suggesting that this cleavage requires an intermediate assembly status of the head structure to occur. Thus, in this model two layers of quality control (body and head structure assembly) allow that only properly assembled SSU are matured in the cytoplasm.

In conclusion, these results suggest a model where the interaction between the SSU r-proteins and their target rRNA are stabilised in the course of the *in vivo* SSU biogenesis. First, most SSU r-proteins interact weakly with the earliest pre-rRNAs. Apparently these weak interactions are - for some - of functional relevance, since *in vivo* depletion of a subset of r-proteins inhibits cleavages of these early pre-rRNAs. Second, SSU r-proteins are further

stabilised within the 20S rRNA containing particles, and association gets more intimate in mature ribosomal subunit. This stabilisation process is presumably linked to rRNA cleavages and structural rearrangements. Finally, while body formation is linked to early rRNA processing events, the status of assembly of the head structure determines, at least to some extent, the competence of the pre-SSU for D site cleavage.

Do ribosome biogenesis factors facilitate r-protein assembly?

Interestingly, rRNA processing defects seen after SSU ribosome biogenesis factor depletion are very similar to those observed after depletion of individual rpS. More than 150 ribosome biogenesis factors were described in the yeast *S. cerevisiae* mainly on the basis of rRNA processing defects in depletion experiments. However despite many efforts the exact function(s) of most of these factors remain elusive.

Among their possible role, it was hypothesised that some of them could be assembly factors, meaning that they would facilitate either the assembly of r-proteins and/ or participate directly or indirectly in rRNA structural rearrangements facilitating the assembly process. Thus the consequence of depletion of one of these proteins would result in a similar rRNA processing phenotype.

For instance, in the yeast *S. cerevisiae*, few ribosome biogenesis factors were described or suggested to be r-proteins assembly factors.

Several ribosome biogenesis factors namely Nep1p, Rrp7p, Yar1 and Tom1p were suggested to be assembly factors of rpS19, rpS27, rpS3 and rpS0 respectively (Baudin-Baillieu et al., 1997; Tabb et al., 2001; Loar et al., 2004; Buchhaupt et al., 2006). Over-expression of the above respective rpS is able to suppress the lethal or slow growth phenotype of the depletion or knockout of the corresponding ribosome biogenesis factor mentioned. The authors of these analyses were suggesting that the increased r-protein amount could enhance the r-protein association with rRNA, and thus partially or completely compensate the loss of function phenotype of the respective ribosome biogenesis factors studied. However, this hypothesis based on genetic analysis remains to be further characterised.

It was also proposed at least for one large subunit r-protein that a ribosome biogenesis factor – Rrs1p – recruits rpL11 onto the pre-60S subunit (Nariai et al., 2005).

Finally, in the course of this work it was suggested that phosphorylation depending on the kinase Hrr25p and dephosphorylation of rpS3 can trigger salt stable assembly of this r-protein and the subsequent formation of the beak structure a structural landmark of the SSU head structure (Schafer et al., 2006).

With the help of the tagged r-proteins strategy mention above, it is now possible to directly determine the status of r-protein assembly in ribosome biogenesis mutants in order to determine which of these factors are *bona fide* r-protein assembly factors.

Alternatively, function and recycling of ribosome biogenesis factors can depend on the presence or absence of r-protein-rRNA dependent structural landmark. For example, it was shown that assembly of rpL10 is required for the release of the LSU export adapter, Nmd3p, from this subunit (Hedges et al., 2005; West et al., 2005).

Therefore, future work will have to determine how both r-proteins and ribosome biogenesis factors assembly status correlate *in vivo*.

1.1.2. The roles of r-proteins in export of the SSU.

The results described above (results 2.) suggest that a certain r-protein assembly status is required to render the SSU competent for nuclear export.

Interestingly, formation of a stable assembly intermediate of the SSU head structure strictly depends on the presence of rpS5 (and is not required for early processing events). In addition rpS5 is required for nuclear export of the SSU-precursors. This indicates that the formation of a stable assembly intermediate of the SSU body in the absence of rpS5 occurs in the nucleus but is not sufficient to enable a consecutive translocation through the nuclear pores. Moreover, most individual r-proteins located in the head-like structure are required for (efficient) translocation of pre-ribosomes through the nuclear pores (see Fig. 28). Furthermore, the analysis of the assembly status after rpS15 depletion reveals an additional assembly intermediate of the eukaryotic SSU head-like structure. Interestingly rpS15 depletion shows a strong 20S rRNA nuclear export delay (Leger-Silvestre et al., 2004 and this work), suggesting that ongoing formation of a stably assembled head-like structure has a strong impact on the ability of the SSU to leave the nucleus.

Finally, ribosomal exportin Crm1p inhibition results in an almost complete block of late (20S) rRNA precursor export to the cytoplasm but allows stable association of all r-proteins that form a head-like, rpS5-dependent structure. All together these results suggest that the establishment of a head-like structure of the SSU within the nucleus represents a necessary prerequisite to allow efficient Crm1p-mediated passage through the nuclear pores.

Thus, it is possible that the head-like structure can serve as a docking domain for the export machinery and/ or for factors facilitating the export process. Alternatively, the head-like feature can be important for either shielding the pre-40S particle when it is exported through the nuclear pore complex or making it compact enough for the passage.
Accordingly the lack of a single r-protein of the head-like structure can result in an incompletely folded head assembly intermediate. This could explain why export of 20S rRNA is significantly retarded or inhibited in mutants in which single r-proteins forming the head-like structure were depleted.

Interestingly, two r-proteins – rpS0 and rpS2 – show reduced 20S rRNA export efficiency after their respective depletion. Surprisingly, these r-proteins do not assemble according to an rpS5 dependent head-like structure. Thus suggesting that these r-proteins are required but not sufficient to trigger nuclear export.

In order to understand the putative role of these body-like r-proteins in SSU export, it remains to determine how assembly of these r-proteins and in a more general way how formation of the body-like structure influences the assembly of the head-like structure.
It is possible that *in vivo* assembly status of the body-like structure controls formation of the head-like structure. Alternatively, if the head-like structure assembles independently of the body-like structure, rpS0 and rpS2 could serve as a platform facilitating directly or indirectly the export process. Thus in this model two layers of quality control (body and head structure assembly) allow that properly assembled SSUs are efficiently released through the nuclear pore and further matured in the cytoplasm.

1.2. Comparison between pro- and eukaryotic SSU r-protein functions.

1.2.1. R-proteins are crucial for cell viability in eubacteria and eukaryotes.

So far in *E. coli* only 6% (302/4709 tested) of the genes were described to be essential for cell viability (see profiling of *E. coli* chromosome at www.shigen.nig.ac.jp/ecoli/pec/) and 18.7% (1105/5916 tested) in *S. cerevisiae* (Giaever et al., 2002). R-proteins belong to these groups of important proteins required for cell viability.

The r-protein families of both ribosomal subunits can be divided into two large groups: those conserved between bacteria, archaeabacteria, and eukaryotes and those only common in archaeabacteria and/ or eukaryotes. Fifteen r-proteins of the 32 yeast SSU r-proteins are universally conserved in all three evolutionary domains; 13 are conserved between archaeabacteria and eukaryotes and only four have no counterparts in prokaryotes (see introduction Fig. 1C and Lecompte et al., 2002).

Recent systematic deletion analysis of SSU r-proteins in *E. coli* indicates that 71.4% (15/21) of the SSU r-proteins are essential for cell viability (Bubunenko et al., 2007) while at least 87.5% (28/32) of SSU r-proteins are essential in *S. cerevisiae* (see results 1.1., *Saccharomyces* genome database at http://www.yeastgenome.org/ and Tabb-Massey et al., 2003).

Among the universally conserved SSU r-proteins, 73.3% (11/15) are essential for cell viability in *E. coli* and at least 93.3% are essential for cell viability in *S. cerevisiae* (14(+1?)/15). The two non-essential SSU r-proteins described in *S. cerevisiae* (rpS12 and rpS25A/B) are eukaryote specific r-proteins.
Thus in eukaryotic cells, by far most r-proteins are essential for growth, indicating that besides participating in the formation of a stable ribosomal subunit structure, they have maybe acquired important additional roles.

Interestingly, *in vivo* depletion of most of the eukaryotic SSU r-proteins inhibits production of mature SSU. Therefore, it appears that the primary essential function of SSU r-proteins is to control the assembly/ processing of the SSU, to ensure that only properly assembled (functional) SSU are engaged in translation.

1.2.2. Comparison between *in vitro* and *in vivo* r-protein assembly.

1.2.2.1. R-protein – rRNA interactions are stabilised in the course of ribosome assembly.

The results described above (results 2.) suggest a model where the interaction between the SSU r-proteins and their target rRNAs are stabilised in the course of the *in vivo* SSU biogenesis.

Interestingly, this observation is in agreement with the structural compaction observed during *in vitro* assembly of SSU in *E. coli*.

Although, direct measurement of the strength of interaction between rRNA and r-proteins during *in vitro* reconstitution experiments were not unambiguously assayed in *E. coli*, several studies clearly demonstrated that the assembly of r-proteins is accompanied by structural rearrangements (Powers et al., 1993; Powers and Noller, 1995b; Holmes and Culver, 2004; Holmes and Culver, 2005; Dutca et al., 2007). Furthermore, reconstitution experiments followed by electron microscopy revealed a clear compaction (reduced radius of gyration) of the ribosomal subunit that correlated with the addition of r-proteins (Mandiyan et al., 1989; Mandiyan et al., 1991). This is in agreement with the analysis of conformational changes in rRNA induced by the *in vitro* assembly of individual SSU r-protein primary binders (Powers et al., 1993; Powers and Noller, 1995b; Holmes and Culver, 2004; Holmes and Culver, 2005; Dutca et al., 2007). Many of these studies indicate that some structural rearrangements are temperature dependent, suggesting that *in vivo* additional factors like ribosome biogenesis factors could trigger the formation of the correctly folded intermediates.

Finally, it was shown in yeast that the co-transcriptionally associated SSU processome with nascent rRNA undergoes several structural rearrangements (Osheim et al., 2004 see also discussion & perspectives 1.1.1.).

Most probably assembly of r-proteins is linked to conformational rearrangements leading to local stabilisation of the r-protein-rRNA complex. The induced structural arrangement and/ or stabilisation could then be a prerequisite to allow further assembly of the following dependent r-proteins which can also in turn further stabilise structural rearrangement.

1.2.2.2. Hierarchical assembly of r-proteins.

Previous *in vitro* studies done in *E. coli* were demonstrating that r-proteins assemble according to a hierarchical order, and were classified accordingly into different groups: primary, secondary and tertiary binding proteins according to their respective requirements to interact with the rRNA (reviewed in Nierhaus, 2004 and see introduction 3.1.1.2.).

In this work, the assembly states of SSU r-proteins after depletion of rpS5, and rpS15 were determined. Interestingly, the stable assembly of a group of SSU r-proteins depends on the presence of rpS5, and to a lower extent on rpS15. S19, the prokaryotic homologue of rpS15, was described to act as a secondary binder in the *in vitro* assembly map of the prokaryotic SSU head structure, downstream of the primary binder S7, the homologue of rpS5. Consistently, *in vivo* analysis presented in this work demonstrates that absence of the primary binder-homologue rpS5 affects assembly of the secondary binder homologue rpS15 much more drastically than *vice versa*.

Interestingly, among the group of SSU r-proteins strongly affected by rpS5 depletion, five have homologues in bacteria and their *in vitro* incorporation depends also on primary binder S7 the rpS5 homologue to form the SSU head structure. In agreement, the results presented in this work demonstrate that *in vivo* formation of the body-like structure is independent of the formation of the head-like structure (Samaha et al., 1994; Agalarov et al., 1998; Agalarov et al., 1999).

Altogether, these results suggest that despite the apparent drastic increase in number of essential accessory factors and structural components introduced in the *in vivo* context of eukaryotic ribosome biogenesis, general principles of prokaryotic *in vitro* assembly apply also to *in vivo* eukaryotic ribosome assembly.

Surprisingly, rpS0, the homologue of eubacterial S2, does not strictly depend on rpS5 in its stable interaction with precursor rRNA, as it would be expected by the eubacterial *in vitro* assembly map (Mizushima and Nomura, 1970; Nomura and Erdmann, 1970; Nierhaus, 2004; Talkington et al., 2005). However this difference in assembly dependence correlates with the reduced contact interface between the eukaryotic rpS0 and the SSU head structure (see results Fig. 32B to Fig. 32C). In addition, previous *in vitro* reconstitution of the head structure alone demonstrated that the rpS0 homologue S2 was poorly recovered in this condition (Samaha et

al., 1994; Agalarov et al., 1998; Agalarov et al., 1999). Furthermore S2 *in vitro* assembly was also shown to be dependent on both body and head domain assembly (Mizushima and Nomura, 1970; Held et al., 1974).
Apparently, in yeast *in vivo* assembly of rpS0 does not depend on the assembly of a head-like structure. It remains to determine how body-like structure formation influences rpS0 assembly *in vivo*.

Further analysis will be needed to show exactly how both the eukaryotic and the *in vivo* context influence the hierarchy of r-protein assembly. In addition, the 15 SSU r-proteins that have no homologous counterparts in eubacteria have to be positioned in an "*in vivo* assembly map".

1.3. The multiple functions of r-proteins in ribosome biogenesis.

It is possible that r-proteins required for early rRNA processing can also participate in nuclear export, in cytoplasmic rRNA processing, or in ribosome function (see below). However, because their first essential role is tightly linked to early maturation steps, they could not be assayed for other functions in this screen. As an example rpS14 was found to be involved in early rRNA processing (Moritz et al., 1990; see also Fig. 28) and its carboxy-terminal extension was also recently described to play an important role in late cytoplasmic rRNA processing (Jakovljevic et al., 2004).
In addition, some r-proteins (e.g. rpS3) are required for both efficient SSU export and late cytoplasmic maturation. Therefore, it would be interesting to uncouple these two procesess to understand the molecular requirement for efficient export and late cytosplamic maturation.

With the help of the genetic system developed in this work, future mutational analysis can be performed either to uncouple and/ or to bypass the primary phenotype observed after depletion of the corresponding r-protein.
It will then be interesting to analyse the consequences on SSU assembly status in strains bearing such mutant alleles in comparison to the complete depletion of the respective r–proteins.

1.4. On the importance of co-transcriptional assembly of r-proteins.

It is not clear whether SSU r-protein assembly is or has to be co-transcriptional *in vivo*. According to the "assembly gradient" hypothesis co-transcriptional assembly of r-proteins would facilitate assembly events and could avoid inhibitory rRNA rearrangement(s) (Nierhaus, 2004).

In addition, according to the "assembly gradient" theory (Nierhaus, 2004), it is possible that *in vivo* folding and assembly of 3´domains of the rRNA (head domain) requires the preceding co-transcriptional formation of defined structural intermediates of the 5´ domain (body/ platform).

Miller spread analysis in pro- and eukaryotes suggest that several proteins can associate co-transcriptionaly with the nascent rRNA, into a structure called in eukaryotes «terminal knobs» (Miller and Beatty, 1969; Hofmann and Miller, 1977). Recently it was suggested that this terminal knobs are the SSU processome components (Dragon et al., 2002; Osheim et al., 2004). Furthermore, the appearance of differently shaped terminal knobs on the rRNA transcript suggests that various significant conformational changes in the nascent ribosome are occuring in the course of RNA polymerase I elongation (Osheim et al., 2004).

Interestingly, it was suggested that ribosome assembly and transcription could be functionally coupled and co-regulated (Dragon et al., 2002; Gallagher et al., 2004; Osheim et al., 2004; Schneider et al., 2007)

As shown in this work, most SSU r-proteins can interact weakly with early (post-transcriptional) rRNA precursors. Among the SSU processome components few SSU r–proteins were suggested to be part of this complex (Bernstein et al., 2004). However, the experimental set-up used in these studies cannot distinguish between co- or post-transcriptionally assembled complexes.

It is likely, that at least some of the r-proteins assemble during transcription as it was previously reported for two r-proteins from *Drosophila* (Chooi and Leiby, 1981). Although, it was recently suggested that r-proteins are post-transcriptionally assembled in higher eukaryotes (Kruger et al., 2007).

The crucial question: whether ribosome assembly is functionally coupled with rRNA transcription remains still to be fully answered.

With the help of the conditional strains for the SSU r-proteins constructed in this work, it is now possible to examine whether the structural features observed after Miller spreads are lost

or altered upon r-proteins depletion, which could indicate that some r-proteins co-transcriptionally assemble into this structural feature.
Alternatively, direct localisation by immuno-localisation of r-proteins on Miller spreads (as was reported by Chooi and Leiby, 1981) using the tagged r-proteins described in this work could be a method of choice.
However, the Miller chromatin spreading technique is not a laboratory routine method. Furthermore, so far the above study is the only published example of an immuno-EM analysis on Miller spreads. Thus it is not clear how feasible it is to study co-transcriptional assembly using this methodology.

Another possibility is to use chromatin immuno-precipitation (ChIP) as it was used to demonstrate co-transcriptional assembly of proteins with nascent mRNA (see among others Strasser et al., 2002). However so far, the different attempts performed to co-precipitate rDNA containing chromatin with this method were not successful (data not shown). Finally it is possible to analyse whether RNA Polymerase I subunits can be coimmuno-precipitated with Flag-tagged SSU r-proteins.

From the analysis presented in this work it can be concluded that formation of a body-like structure is independent on the stable assembly of a head-like feature. To gather further evidence for the existence of an *in vivo* "assembly gradient" it is possible to investigate by similar means whether formation of the head-like structure requires the previous folding of the body-like feature. The final goal is then to determine whether and how the flux of rRNA synthesis influences the folding of major (pre)-ribosomal morphological features.

1.5. R-protein assembly and rRNA modifications: a connection?

The exact function(s) of the numerous rRNA modifications are still unclear, but these modifications were suggested to mainly participate in the translation process (see introduction 2.2.1.3.). However, how ribosome assembly and rRNA modifications are co-ordinated is still unclear.

Recently, the various rRNA modifications could be positioned within the 3D structure of the ribosome. Strikingly, modifications are apparently absent from the known areas of r-proteins contact with the rRNA. However the eukaryotic ribosome structure is lacking all the

eukaryote specific rRNA and r-proteins counterparts, thus it is difficult to completely exclude the proximity of rRNA modifications and r-proteins contacts.
Furthermore, the rRNA modifications are localised in regions that can be highly structured (e.g. secondary structure like hairpin, double stranded RNA). In eukaryotes, snoRNP dependent modification in double stranded rRNA regions must occur prior to the final stabilisation of these structures in the subunit. In addition some r-proteins have contact with the rRNA upstream or downstream of these structures, suggesting that a coordination of the two processes is necessary.

The following questions emerge from these observations: Do rRNA modifications influence proper r-proteins assembly? Does r-protein assembly influence rRNA modifications?

It was suggested that efficient SSU assembly *in vitro* requires modified rRNA (Cunningham et al., 1991). Moreover, *in vivo* analyses of conditional mutants affecting the enzymatic activity of the respective modifying enzymes in yeast indicate that the lack of rRNA modifications affects cell growth and that the total amount of mature ribosomal subunits produced is reduced (Tollervey et al., 1993; Zebarjadian et al., 1999). Since it cannot be excluded that these phenotypes correlate with translation defects it remains unclear whether rRNA modifications facilitate (stable) r-protein assembly *in vivo*.

Furthermore, it was shown *in vitro* in *E. coli* that two adjacent modified rRNA residues are modified in a succesive way (Weitzmann et al., 1991). Modification at G967 can occur on naked rRNA template while in contrast modification at C966 is inhibited. In addition assembly of S7 and S19 is sufficient to allow the m^2G966 rRNA modification and to inhibit the m^5C967 modification. Previous study also indicates that the assembly of S7 and S19 induces local conformational changes of the loop containing these two modified residues (Powers et al., 1988). Interestingly, although the rRNA modification mechanism is different between eubacteria and eukaryotes, these modified sites are conserved. Furthermore in eukaryotes there is an additional rRNA modification in the proximity of C966 and G967. Since the prokaryotic r-proteins S7 and S19 homologues in yeast, rpS5 and rpS15, share similar assembly characteristics, it would be interesting to determine the pre-rRNA modification status at these positions after *in vivo* depletion of these repective r-proteins.

Future work will have to determine whether there is a functional relationship between r–protein assembly and rRNA modifications. In addition understanding how rRNA modification status and r-protein *in vivo* assembly correlate will facilitate to establish a precise temporal map of the different ribosome assembly events.

1.6. Localisation of eukaryotic SSU r-proteins in the ribosome structure.

As indicated earlier (see introduction 1.2.2.), eukaryotic SSU structure was obtained with the help of cryo-electron-miscroscopy analysis and homology modelling. However, these studies did not allow to position SSU r-proteins that have homologous counterpart only in archaeabacteria and/ or eukaryotes, which represent around 50% of the r-proteins of the eukaryotic SSU. In addition, early protein topography determined by protein-protein chemical cross-linking from the eukaryotic SSU are difficult to interpret because of inconsistent nomenclature usage, and loss of some proteins during sample preparation/ analysis (Yeh et al., 1986).

Although, the analysis presented in this work does not allow the exact positioning of all these r-proteins in the SSU structure, it might be possible to deduce their position in the structure according to their respective assembly behaviour.

For example, it was recently suggested that rpS10 and rpS19 contact the rRNA/ r-protein counterparts forming the head structure of the SSU (Valasek et al., 2003; Buchhaupt et al., 2006 respectively). It was shown by two hybrid analysis that rpS10 interact with the eIF3 sub-complex and rpS0. On the basis of the rpS0 and eIF3 complex localisation, it was proposed that rpS10 could be located in an additional electron density spanning from rpS0 towards the head-structure (Valasek et al., 2003). This suggests that rpS10 has probably some contacts with the head domain. Interestingly, the results presented in this work show that stable assembly of rpS10 and rpS19 depends on the formation of the head-like structure.

These results remain to be confirmed by more direct structural analysis. So far no crystallisation procedure for archaeabacterial or eukaryotic SSUs were reported. The collection of tagged rpS described in this work could serve as a starting point to perform localisation by immuno-EM approach as decribed previously (among others Tischendorf et al., 1974a; Tischendorf et al., 1974b; Tischendorf et al., 1975; Tsay et al., 1994). Finally, two

rpS were found to be not essential for cell viability. Therefore it is possible to analyse their localisation within the SSU by differential analyses of the electron density maps of wildtype and mutant ribosomal subunits (Halic et al., 2005).

2. R-proteins and translation.

2.1. R-proteins as modulator of ribosome function.

As indicated earlier, (introduction 3.2.) the ribosome is a ribozyme but ribosome function depends on cooperation between the rRNA and r-proteins.

In this work most of the SSU r-proteins were shown to be primarily required for SSU biogenesis, with the exception of the non-essential SSU r-proteins – rpS12 and rpS25 - and the three essentials SSU r-proteins — rpS7, rpS30, and rpS31— that seem to participate in activities that are more related to ribosome function.

Previous studies indicated that rpS9 and rpS2 are important for translation accuracy (Alksne et al., 1993; Synetos et al., 1996; Stansfield et al., 1998) while loss of function analysis performed in this work suggests that these proteins are in addition required for early and late biogenesis steps respectively (see Fig. 28). Since, r-proteins can play a role in several steps of SSU biogenesis but also in ribosome function, further mutational analyses are required to study their function in the mature ribosomal subunit.

2.2. The ribosome as a regulator of gene expression: “the ribosome filter hypothesis”.

Recently, the “ribosome filter hypothesis” was suggested (Mauro and Edelman, 2002). This hypothesis suggests that ribosomes are regulatory elements that affect the translation of particular mRNAs by binding differentially to them. The filter hypothesis also predicts that structural differences in ribosome populations and in mRNA populations may affect the filter. If this assumption is valid, one would expect that different ribosomes (for example, those that differ in their rRNA and/ or ribosomal protein composition/ modifications) might bind to particular mRNAs to different extents, thus altering the relative efficiencies of translation.

Interestingly, there is some evidence that ribosomes are not homogenous RNPs, but instead are rather heterogeneous macromolecular complexes.

Heterogeneity of ribosomal subunits has different reasons:

The genetic organisation of the ribosomal components itself participate in ribosome heterogeneity. The rRNA genes are often transcribed from multi-gene families and heterogeneity can be caused by various polymorphisms (nucleotide substitution, or deletion). Thus these variations in rRNA sequence can contribute to ribosome heterogeneity.
Another possible source of heterogeneity is the existence of genetic variants of the r-proteins in eukaryotes. Fungi and plants are among the organisms presenting the most important number of possibilities in terms of variation of r-proteins composition since many of the r-proteins coding genes exist in multiple copies (Nakao et al., 2004). However this r-protein heterogeneity is not restricted to plants and fungi, for example in human cells two variants for rpS4 (92% identity) encoded on the X and the Y chromosomes have been described.

In addition, rRNA and r-protein modifications can be a source of ribosome heterogeneity.
It is known since a long time that rRNA is strongly modified, however it is not clear whether all modified residues are quantitatively modified *in vivo*. If not this clearly raises the question whether rRNA modification patterns could contribute to ribosome heterogeneity. Finally, r–proteins are also modified (phosphorylated, methylated…) (Kruiswijk et al., 1978a; Kruiswijk et al., 1978b), and their stoichiometry in the subunit can be also a source of heterogeneity. One remarkable example is the ribosomal subunit dynamic observed in *D. discoideum*, in which the ribosome r-protein composition is quantitatively and qualitatively different depending on the cellular differentiation state (Ramagopal and Ennis, 1981; Ramagopal, 1992; Mauro and Edelman, 2002).

Finally, another possibility of ribosomal subunit heterogeneity comes from rRNA processing.
At least, in the yeast *S. cerevisiae* the alternative processing pathway lead to the unbalanced formation of $5.8S_S$ and $5.8S_L$ rRNA (see introduction 2.2.2.3.) which can also contribute to heterogeneity.

In this work, it was shown that two eukaryotes specific r-proteins are not essential for cell viability in the condition tested. However, it is not known whether these r-proteins when missing are not responsible for mRNA translational control. A way to analyse this possibility would be to analyse by micro-array, which mRNAs are associated with translating ribosomes containing these r-proteins.

Finally, preliminary analysis on the stoichiometry of SSU r-proteins suggests that at least two r-proteins are sub-stoichiometrycally represented in the mature SSU. These preliminary results suggest that these two r-proteins could be interesting candidates participating in a differential mRNA translation control.

As mentionned before, a micro-array analysis could reveal which mRNAs are associated with ribosomes containing these sub-stoichiometrically represented r-proteins.

3. Conclusion: SSU r-proteins *in vivo* functions and assembly pathway.

In conclusion, this work established a genetic system allowing to study (1) the *in vivo* role of SSU r-proteins in ribosome biogenesis and ribosome function, and (2) the principles of eukaryotes *in vivo* r-proteins assembly pathway.

The different analyses performed with these tools show that most individual SSU r-proteins are required (1) for cell growth (2) for efficient production of mature 18S rRNA (3) for different steps of the SSU biogenesis, and can be grouped according to their rRNA processing defect phenotype after depletion. Interestingly, all the known pre-rRNA intermediates described in wildtype and ribosome biogenesis mutant strains were observed after depletion of individual SSU r-proteins, suggesting that individual SSU r-proteins participate in each step of SSU maturation. The results indicate that SSU r-proteins can be further grouped according to the 20S rRNA maturation defects shown after depletion. (4) SSU r-proteins rpS5, rpS18, and rpS19 are required for late nuclear processing and/ or quality control steps leading to nuclear retention of 20S rRNA. (5) Depletion of SSU r-proteins rpS0, rpS2, rpS3, rpS10, rpS15 and rpS26 appear to first affect the export rate/ competence of the SSU and then the late maturation steps of conversion of 20S pre-rRNA into mature 18S rRNA. Furthermore, (6) when one of the SSU r-proteins rpS7, rpS20, rpS30, or rpS31 is lacking, a significant amount of neo-synthesised 20S rRNA reaches the cytoplasm. (7) In strains depleted in rpS7, rpS30, and rpS31, some of the cytoplasmic 20S rRNA can be further processed into mature 18S rRNA.

The second part of this work shows that (1) most SSU r-proteins interact weakly with the earliest rRNA precursor (2) these weak interactions are - for some r-proteins - of functional relevance. (3) SSU r-proteins interactions with pre-rRNA are stabilised in the course of the SSU assembly. (4) This stabilisation apparently correlates with rRNA maturation events. (5) Formation of a stable assembly intermediate of the body-like, but not of the SSU head-like structure is tightly linked to early processing events (A0, A1, and A2 cleavages). (6) Formation of a stable assembly intermediate of the SSU head-like structure depends on the presence of rpS5 (and is not strictly required for early processing events). (7) Key aspects of the *in vivo* assembly of eukaryotic r-proteins into distinct structural parts of the SSU are similar to the *in vitro* assembly pathway of their prokaryotic counterparts (e.g. body-like

formation independent from head-like formation, absence of a primary binder-homologue affects assembly of the secondary binder-homologue much more drastically than *vice versa*). (8) Formation of a stable assembly intermediate of the SSU body occurs in the nucleus but is not sufficient to enable a consecutive translocation through the nuclear pores. (9) Formation of a stable assembly intermediate of a SSU head-like structure precedes and is required for (efficient) nuclear export. (10) Final cytoplasmic maturation events, which then lead to the mature SSU, require stable assembly of the SSU head-like structure and are accompanied by further structural rearrangement (e.g. beak formation).

MATERIALS & METHODS

All the vectors and yeast strains used in this work are listed and described in Table 1 and 2 respectively.

1. Microbiology work.

1.1. Work with *Escherichia coli.*

All the *Escherichia coli* strains were incubated, except indicated, at 37°C.

1.1.1. Media.

E. coli strains were grown on Luria-Bertani (LB) media [1% NaCl (Merk) 1% Bacto-Trypton (BD), 0,5% Yeast Extract (BD)]. For solid media 2% Bacto-agar (BD) was added.

Ampicilin resistance selecting media (LB-amp):
The ampicillin belongs to the group of β-Lactam antibiotics and inhibits the final stage of bacterial cell wall synthesis, which leads to cell lysis.
E. coli strains expressing the β-lactamase encoded by the BLA gene are resistant to β-lactam antibiotics and were selected on LB media containing 100 μg/ml of ampicilin (Roth)

1.1.2. Competent cells for electroporation.

XL1-blue and DH5α strains were used as a host for amplification of plasmid DNA.
In order to increase the efficiency of plasmid DNA uptake, *E. coli* competent cells for electroporation were prepared. The exposure to an electrical charge of *E. coli* cells induces the formation of transient membrane pores through which DNA molecules can pass (Neumann and Rosenheck, 1972).
Cells are grown in SOB (2% Bacto-Trypton, 0.5% Bacto-Yeast extract, 8.55 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, pH 7) at 37°C to mid-log phase (OD_{600}= 0.35 – 0.6), chilled on ice for 15 min and centrifuged. Cells are washed 3 times with ice-cold sterile water to reduce the ionic strength of the cell suspension. The cells are resuspended in 10% sterile glycerol (on average 1-3.10^{10} cells per ml), aliquoted (50 μl) and store at –80°C.

1.1.3. Transformation with plasmid DNA.

Electro-competent cells (see materials & methods 1.1.2.) are thaw on ice, incubated for 5 min with DNA on ice, and transfer into electroporation cuvette (Biorad). Cells are exposed to a short high voltage discharge (2.5 kV, 5-6 ms) using a Biorad Micropulser.
Cells are resuspended in LB media, incubated for 1 h at 37°C and platted on the appropriated selection medium (in this work on LB-Amp plates see materials & methods 1.1.1.) and grow over-night at 37°C.

1.1.4. Purification of plasmid DNA (mini- and midi-preparation).

Isolation of plasmid DNA from bacteria was performed according to the manufacturer using the following kits: FastPlasmid Mini (Eppendorf), Plasmid Mini- and Midiprep kit (Qiagen). Briefly, cells are lysed, and plasmid DNA is isolated from the lysate by DNA trapping on a matrix. The plasmid DNA is further washed with alcohol-based solution, and eluted from the matrix with Milli-Q grade water.

1.1.5. Long-term storage.

Logarithmically growing bacterial strains were stored at –80°C in culture media supplemented with 16.5% (w/v) glycerol.

1.2. Work with *Saccharomyces cerevisiae*.

All the yeast strains were incubated, except indicated, at 30°C.

1.2.1. Media.

1.2.1.1. "Full media" or YP media.

YP media is composed of 1% Yeast extract, 2% Bacto-peptone (all from BD) supplemented with 2% of an appropriate carbon source. In this work either D (+) glucose (Merk) or D (+) galactose (Sigma-Aldrich) (all microbiology grade) were used as carbon sources.
For culture on solid media 2% Bacto-agar (BD) was added.

1.2.1.2. Synthetic media.

Complete synthetic media is composed of 6.7 g of yeast nitrogen base containing the required salts, vitamins, and a nitrogen source (Sunrise Science) supplemented with the appropriate amino acids and nucleotides mixture (Sunrise Science) and a carbon source (see materials & methods 1.2.1.1.) to allow cell growth and/ or selection.
For culture on solid media 2% Bacto-agar was added.

Amino acids and nucleotides used in synthetic media (final concentration):
Adenine 10 mg/L; L-Arginine HCL 50 mg/L; L-Aspartic acid 80 mg/L; L-Histidine HCl 20□mg/L; L-Leucine 100 mg/L; L-Lysine 50 mg/L; L-Methionine 20 mg/L; L-Phenylalanine 50 mg/L; L Threonine 100 mg/L; L-Tryptophan 50 mg/L; L-Tyrosine 50 mg/L; Uracil 20□mg/L; L-Valine 140 mg/L (single component were all purchase from Sigma)
Amino acids and nucleotides pre-made drop out mixtures were all purchased from Sunrise Science and used as indicated by the manufacturer.

1.2.1.3. Sporulation media.

Yeast diploid cells were inoculated on sporulation media containing 0.25% yeast extract, 1.5% potassium acetate, 0.05% glucose, 2% agar for minimum three days.

1.2.1.4. 5-fluoro-orotic acid (5-FOA) selection.

The 5-FOA is converted by the orotidine-5'-phosphate decarboxylase encoded by the URA3 gene (in *S. cerevisiae*) into a toxic substance the 5' fluorouridine monophosphate, which severely limit cell growth (Boeke et al., 1984).
Therefore this molecule is really useful to characterise loss (of function) of the URA3 gene in a yeast population in different kind of assay.

5-FOA containing medium was made with synthetic media (see materials & methods 1.2.1.2.) containing the required drop out mix and carbon source supplemented with 1 g/L of 5-fluoro-orotic acid, monohydrate (Toronto Research Chemicals).

1.2.1.5. Geneticin selection media.

Geneticin or G418 is an aminoglycoside antibiotic that irreversibly binds the 80S ribosome and inhibits protein synthesis in eukaryotic and prokaryotic cells. The geneticin effect can be inactivated by the expression in pro- or eukaryotes of the aminoglycoside 3'–phosphotransferase encoded by the KanMX gene that inactivates the aminoglycoside inhibitory effect through phosphorylation of the geneticin molecule (Jimenez and Davies, 1980; Bar-Nun et al., 1983; Eustice and Wilhelm, 1984a; Eustice and Wilhelm, 1984b).

Selection is commonly made on full media (materials & methods 1.2.1.1.) supplemented with 200 µg/ml of G418 sulfate (Difco) (Wach et al., 1994). The geneticin resistance selection can be also made on a modified synthetic media using proline 1 g/L (Sigma) as nitrogen source instead of ammonium sulfate (Cheng et al., 2000).

1.2.1.6. Cycloheximide containing media.

Cycloheximide is a protein synthesis inhibitor interfering in eukaryotes with the peptidyl transferase activity, thus blocking elongation (Stanners, 1966; Munro et al., 1968; Baliga et al., 1969; Obrig et al., 1971).
For cycloheximide resistance/ sensitivity assay, serial dilutions of logarithmically growing cells were spotted on YP-glucose media (materials & methods 1.2.1.1.) supplemented with 0.5 µg/ml cycloheximide (Roth).

1.2.1.7. Paromomycin containing media.

Paromomycin is an aminoglycoside antibiotic which bind to the A-site of the ribosome, and interferes with the loading of aminoacyl-tRNA, inducing amino acids misincorporation during protein synthesis (Fourmy et al., 1996; Burman et al., 2003).

For paromomycin resistance/ sensitivity assay, serial dilutions of logarithmically growing cells were spotted on YP-glucose media (materials & methods 1.2.1.1.) supplemented with increasing amount (from 0.5 to 10 mg/ml) of paromomycin sulfate (Fluka).

1.2.2. Yeast competent cells.

Treatment of yeast cells with alkali cations (e.g. Li^+, Cs^+, K^+ see Ito et al., 1983) is effective to induce competence of yeast cells to uptake linear and circular DNA molecules. Efficiency of transformation can be further increased in the presence of other chemicals (see materials & methods 1.2.3.).

Fifty ml of mid-log phase growing cells (OD_{600} 0.5-0.7) are centrifuged and washed once with sterile water. Yeast cells are further washed twice with sterile filtrated lithium-sorbitol buffer (100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1 M sorbitol, buffered to pH 8 with acetic acid). Cells are then resupended in 360 µl lithium-sorbitol buffer and 400 µg of heat denatured salmon sperm DNA (10 mg/ml - Invitrogen), aliquoted (50 µl) and store at –80°C.

1.2.3. Transformation with DNA.

Yeast competent cells are thaw on ice and incubated with circular (100-500 ng) and/ or linear (on the µg range for efficient homologous recombination) DNA molecules. After addition of 6 volumes of LitPEG buffer [(40% (w/v) PEG3350 (Sigma), 100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, buffered to pH 8 with acetic acid and sterile filtrated)], cells are incubated 30 min at room temperature. DMSO ($1/9^{th}$ volume- Merck) is added and the cells are incubated at 42°C for 15 min. Cells are spun down and resuspended in sterile water and platted on the appropriated selection medium.

1.2.4. Preparation of high molecular weight yeast genomic DNA.

An overnight yeast culture is centrifuged. Peletted cells are washed with water and resuspended in spheroplasting buffer [(0.9 M sorbitol, 0.1 M EDTA pH8, 50 mM DTT (Sigma), 0.66 U/ml Lyticase (ICN Biomedicals)] and incubated 30 min at 37°C in order to digest the cell wall of the yeast cells, which are then further called spheroplast (yeast without cell wall). Spheroplasts are lysed in 50 mM Tris-HCl pH8, 50 mM EDTA, 1% SDS for 5 min at 65°C and chilled on ice after addition of potassium acetate to a final concentration of 1.25 M.

After centrifugation of the lysed cells, the supernantant is collected.

The soluble nucleic acids are precipitated by addition of isopropanol to a 50% (v/v) final concentration. Nucleic acids pellet is washed with 70% ethanol, dried, resuspended in water containing 10 μg/ml RNase (Boehringer Mannheim) and incubated for 30 min at 37°C.
Quality of the prepared yeast genomic DNA is further assay by agarose gel electrophoresis (see materials & methods 2.1.1.).

Purified high molecular weight yeast genomic DNA from yeast strain S288C (Invitrogen) was also used.

1.2.5. Mating of yeast haploid strains.

Haploid yeast strains of opposing mating type (Mat a and α), were grown independently over-night. Cells were mixed, and incubated from 10-24 h to allow mating. Diploid cells were isolated using dominant selection marker specific for each initial haploid strain. Alternatively, fusing cells or "shmoo" can be identified and isolated by micromanipulation few hours after mixing the two haploid strains.

1.2.6. Sporulation of diploid cells.

Starvation of nitrogen and carbon source induces sporulation of diploid strains. In this condition diploid strains undergo meiosis division resulting in the formation of 4 spores (tetrad) contained in an ascospore. Cells were incubated minimum 3 days on sporulation media (see materials & methods 1.2.1.3.).

1.2.7. Tetrad analysis.

Sporulated cells were resuspended in sterile water containing zymolyase (10 mg/ml) and incubated 5 min in order to open the cell wall of the ascospore. The spores were isolated by micromanipulation using a MSM Singer micromanipulator. Isolated offspring were genetically characterised by selection on the appropriated media.

1.2.8. Long-term storage.

All yeast strains were stored in duplicate. Logarithmically growing yeast strains were stored at –80°C in culture media supplemented with 16.5% (w/v) glycerol.

2. Work with nucleic acids.

DNA and RNA samples were all stored at –20°C except mentioned.

2.1. Work with DNA.

2.1.1. Native agarose gel electrophoresis.

Agarose gel electrophoresis was used to separate DNA fragments of different lengths. In this work, electrophoresis was performed routinely with 1.0-1.2% (w/v) agarose (Invitrogen), 1X TBE (90 mM Tris-borate, 1 mM EDTA) gels containing 0.2 µg/ml ethidium bromide, and 1X TBE as electrophoresis buffer. To determine the lengths of the fragments, 250 ng of DNA standard [1 kb ladder and/ or 100 bp ladder (NEB)] were used, both in a concentration of 50 µg/ml in 1X DNA loading buffer (6.7% sucrose; 0.04% bromophenol blue and xylene cyanol FF – Serva and Sigma respectively).

2.1.2. Polymerase Chain Reaction (PCR).

The polymerase chain reaction allows the exponential amplification of DNA fragments *in vitro*. The isolated DNA fragments can be further used in various experiments such as cloning. The principles of PCR are widely discussed in the literature and applications are extremely versatile.

In this work, PCR were performed with yeast genomic DNA (100-500 ng) or plasmid DNA (10-100 ng) as templates in 50-100 µl reaction [20 mM Tris-HCl, 10 mM $(NH4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 25 µM of reverse and forward primers, 25 µM dNTP, and 2-5 U Taq Polymerase (NEB)].

The main PCR program used in this work was 1 cycle 95°C for 5 min; 45°C for 2 min; 72°C for 2 min followed by 35 cycles 95°C for 1 min; 45°C for 2 min; 72°C for 2 min, and 1 cycle 95°C for 1 min; 45°C for 2 min; 72°C for 10 min.

Samples ($1/10^{th}$) were analysed by agarose gel electrophoresis and subsequently purified with PCR purification kit (Qiagen) according to the manufacturer.

2.1.3. Digestion of DNA with restriction endonucleases.

A variety of prokaryotic restriction endonucleases (NEB) were used to digest DNA in order to prepare defined DNA fragments for cloning or to check for presence and correct orientation of inserted DNA fragments. Restriction endonucleases were essentially used as suggested by the manufacturer.

2.1.4. Purification of DNA.

2.1.4.1. Purification of DNA from solution.

DNA was recovered from aqueous solutions either by ethanol precipitation or by commercial DNA trapping based purification kit.

Several alcohol based nucleic acid precipitation protocols are described in the literature (Sambrook and Russell, 2001). In this work, DNA was precipitated, from aqueous solution, by addition of 2.2 volumes of absolute ethanol and $1/10^{th}$ volume of 3 M NaAc pH 5.8 for minimum 20 min at –20°C. Ethanol depletes the hydration shell from nucleic acids and expose negatively charged phosphate groups. Counter cations (here Na^+) bind the charged groups and reduce the repulsive forces between the polynucleotide chains, allowing the formation of a precipitate. Samples were centrifuged 10 min, 14000 rpm at 4°C. After removal of the supernatant, nucleic acids pellet were dried at room temperature, and solubilised in milli-Q water.

Alternatively, purification of DNA from solution was performed using commercial kits as recommended by the manufacturer (PCR purification kit, or QIAEX II gel extraction kit all from Qiagen).

2.1.4.2. Purification of DNA fragments from agarose gel.

DNA fragments of interest were cut out from agarose gels and eluted using commercial kits following the indications provided by the manufacturer (QIAquick or QIAEX II gel extraction kit all from Qiagen).

2.1.5. DNA ligation.

In order to clone DNA sequences into yeast/ bacterial shuttling vectors, quantity of purified DNA fragments digested with restriction endonuclease(s) was estimated after agarose gel electrophoresis. A three-time excess of insert DNA compare to the vector DNA fragment were incubated in a 15 µl ligase reaction (400U T4 DNA ligase NEB, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 10 mM Dithiothreitol, 25 µg/ml BSA) 1 h at room temperature or over-night at 16°C. One µl of ligation reaction was used for *E. coli* transformation (see materials & methods 1.1.3.).

2.1.6. DNA amount determination.

DNA amount was estimated by agarose gel electrophoresis using a quantitative DNA standard (NEB) as reference or by spectrophotometry method (1 A_{260} ≈ 50 µg/ml) using an Ultrospec 3100 Pro spectrophotometer (Amersham biosciences).

2.1.7. DNA sequencing and oligonucleotides synthesis.

All DNA sequencing and primer synthesis were performed by MWG.
Oligonucleotides used in this work are listed and described in Table 3.

2.2. Work with RNA.

2.2.1. Hot-phenol RNA extraction.

RNA extractions were essentially performed as described previously (Schmitt et al., 1990). This protocol is suited for extraction of total RNA from low amount of samples.
Cell pellets or cell extracts are resuspended in 1 volume of AE buffer (50 mM NaAc pH 5.3, 10 mM EDTA pH 8) and mixed with 1 volume of AE buffer equilibrated phenol (Roth) and 1/10th volume of 10% SDS. The samples are incubated on a thermomixer (Eppendorf) 5 min at 65°C full mix speed (14000 rpm) and chilled on ice for 2 min. Aqueous phase containing the RNAs, are collected, and followed by one phenol extraction (1:1 volume) and one chloroform extraction (1:1 volume). RNAs are precipitated from the aqueous phase at –20°C for 10 min after addition of 2.5 volume of absolute ethanol and 1/10th volume of 3 M NaAc pH 5.3.

Precipitated RNA, when used for denaturing agarose gel electrophoresis, were solubilised in RNA loading buffer (50% formamide, 0.025% xylene cyanol FF and bromophenol blue – Sigma and Serva respectively), denatured for 15 min at 65°C and stored at –20°C. For use with denaturing polyacrylamide gel electrophoresis, samples were further washed with 70% ethanol, and solubilised in 50% deionized formamide (formamide incubated 2 h under mild agitation with Amberlite MB-1 resin - Serva).
For cDNA synthesis, RNA was solubilised in Milli-Q water.

2.2.2. RNA amount determination.

RNA amount was estimated by spectrophotometry method (1 A_{260} ≈ 50 µg/ml) using an Ultrospec 3100 Pro spectrophotometer (Amersham biosciences).

2.2.3. Native agarose gel electrophoresis.

Native agarose gel electrophoresis was used to assay the quality of extracted RNA. Electrophoresis was performed with 1.3% (w/v) agarose (Invitrogen), 0.5X TAE (20 mM Tris-acetate, 0.5 mM EDTA) gels containing 0.2 µg/ml ethidium bromide, and 0.5X TAE as electrophoresis buffer. Extracted RNA solubilised in 50% formamide were denatured as described above prior to loading.

2.2.4. Denaturing agarose gel electrophoresis of high molecular weight RNA.

RNA species over 1000 bases were resolved on denaturating agarose gel (1.3% agarose (Invitrogen), 2% formaldehyde; 0.1 µg/ml ethidium bromide; 1X MOPS buffer: 20 mM Sodium acetate trihydrate, 20 mM MOPS (Fluka), 1 mM EDTA, pH 7). Gels were run for 14–16 h at 40 V in 1X MOPS and 2% formaldehyde electrophoresis buffer.

2.2.5. Denaturing polyacrylamide gel electrophoresis of low molecular weight RNA.

Small sizes RNA (< 1000 bases) were resolved using denaturing polyacrilamide gel electrophoresis (8% acrylamide/ bisacrylamide (37.5/ 1) from Roth), 7 M Urea, 0.5X TBE (45 mM Tris-Borate, 1 mM EDTA).

Gels were pre-run at 100 V for 1 h. Samples were loaded, and run at 100 V for 1-2 h.

2.2.6. Northern Blotting.

Resolved RNAs were transfered and immobilised on positively charged membranes (Positive™ MP-Biomedicals) using different methods (described below). In every case, the RNAs were cross-linked to the membranes by 1 min exposition to UV light source (254/312 nm).

2.2.6.1. Passive capillary transfer.

Prior to transfer, the agarose gels were washed once 5 min in milli-Q water, once 20 min in 0.05 M NaOH to hydrolyse the RNAs and facilitate the transfer of larger RNAs, and were further equilibrated twice 20 min in 10X SSC (1.5 M NaCl, 150 mM sodium-citrate trihydrate).

Transfer of the RNAs from the agarose gel to the membrane is then achieved over-night by drawing the transfer buffer (10X SSC) from the reservoir upward through the gel into a stack of pumping paper. The RNAs are eluted from the gel and deposited onto the positively charged membrane with the help of the buffer stream.

2.2.6.2. Vacuum transfer.

Prior to transfer, agarose gels are treated as described in materials & methods 2.2.6.1. except that gels are incubated only once 20 min in 10X SSC. The RNAs are eluted from the gel onto the positively charged membrane (Positive™ MP-Biomedicals) applying a vacuum of 5 bars for 90 min using a vacuum blotter (Biorad).

2.2.6.3. Electrophoretic transfer.

The RNAs from polyacrylamide gel were transfered onto the membrane (Positive™ MP-Biomedicals) applying an electric current. Transfers were performed using a TransBlot system (Biorad) for 2 h, 40 V at 4°C in 0.5X TBE. After cross-linking, the RNA were stained using a 0.02% methylene blue solution.

2.2.7. Dot Blot.

Positively charge membranes were soaked in 10X SSC, and partially dried. RNA samples were then spotted onto the membrane. After drying, RNAs were cross-linked to the membrane (see materials & methods 2.2.6.).

2.2.8. Probe labelling, hybridisation and detection.

RNA of interest immobilised on solid supports can be detected and quantify in complex mixtures using specific probes with the help of conditions allowing the formation of specific RNA of interest/ probe hybrid.

Probes used in this work are listed in Table 4.

2.2.8.1. Radioactive probe labelling and detection.

5' ends of all oligo-probes were labelled with ^{32}P. Ten pmol of oligo-probe were incubated with 50 µCi of $\gamma^{32}P$-ATP (Amersham), in 1X PNK buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT) and 10U of T4 polynucleotide kinase (NEB) for 30-45 min at 37°C. Reactions were stopped by addition of 1 µl of 0.5 M EDTA pH 8. Labelled probes were purified from the non-incorporated nucleotides by gel exclusion column (Spin6 - Biorad). Incorporated radioactivity was estimated by counting 1 µl of purified-labelled probes using a scintillation counter (1600TR-Packard).

Membranes were pre-hybridised minimum 1 h at 37°C in 50% formamide; 5X SSC; 0.5% SDS; 5X Denhards (1% Ficoll typ400-Pharmacia, 1% Polyvinylpyrrolidone, 1% BSA Fraction V-Sigma). Membranes were then incubated at 37°C over-night after addition of 1-2.10^6 cpm of radiolabelled oligo-probe per blot.

The membranes were washed twice 15 min in 2X SSC, 0.1% SDS and twice 15 min in 1X SSC, 0.1% SDS at 37°C.

Signals were acquired exposing the membrane onto PhosphoImager screen and/ or onto BioMax MS/MR film (Fujifilm).

2.2.8.2. Non-radioactive probe labelling and detection.

Digoxigenin-labelled RNA probes were synthesised using the DIG-Northern starter kit (Roche) as recommended by the manufacturer.

In brief, PCR products containing the T7 promoter followed by the region of interest was used as template for *in vitro* transcription in presence of digoxigenin modified NTP. The resulting probe was stored in aliquot in 50% formamide at –20°C.

Membranes were pre-hybridised for minimum 1 h at 65°C in 50% formamide; 5X SSC; 0.5% SDS; 5X Denhards. Membranes were then incubated at 65°C over-night after addition of 1-2 µl of DIG-labelled probe per blot.
The membranes were washed twice 10 min in 50% formamide; 5X SSC; 0.5% SDS; 5X Denhards and twice 15 min in 0.1X SSC, 0.1% SDS at 65°C.
Blots were washed 3 min at room temperature in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5 and 0.3% N-lauroylsarcosine).
Membranes were incubated 1 h at room temperature in blocking buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5 and 1X blocking reagent (Roche), followed by 30 min incubation with 0.75 U/µl of anti-DIG antibody conjugated to the alkaline phosphatase (Roche) in blocking buffer.
Membranes were washed three times 10 min at RT in washing buffer, and 5 min in reaction buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).
Chemi-luminescent substrate (1% CDP-Star - Roche) was added to the membrane. The signals were acquired using a Fuji LAS Reader 3000 (30 sec increment steps high binning).

2.2.9. Signal quantification.

DIG-labelled and radioactive signals were quantified using AIDA (Raytest) and ImageGauge (Fuji) respectively.

2.2.10. Reverse Transcription PCR (RT-PCR).

Reverse transcriptase experiments were used in order to clone the coding sequences of ribosomal protein genes containing long intron sequence. Single strand cDNA synthesis were carried out as follow:
One µg of total RNA extracted from a wildtype yeast strain was denatured 15 min at 65°C and chilled on ice to prevent inhibition of the reverse transcriptase reaction by secondary

RNA structures. Reaction buffer [(50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 1 mM dNTP, 1.25 mM of mRNA specific reverse primer], 20 U Rnasin, and 200 U M-MuLV reverse transcriptase, were added to the RNA sample (total volume 20 µl). Mixture was incubated 60 min at 37°C, and the reverse transcriptase was inactivated for 5 min at 95°C.

Ten percent of the reverse transcriptase reaction was used as template for PCR with the following settings (5 min 95°C, 35 cycles 30 sec at 95°C, 2 min at 50°C, 3 min at 72°C; and one cycle 30 sec at 95°C, 2 min at 50°C, 10 min at 72°C).

3. Work with proteins.

3.1. Determination of protein concentration.

The Bradford protein assay was used to determine the relative amount of proteins in solution. The Bradford protein assay is a spectroscopic procedure, based on an absorbency shift (from 465 nm to 595 nm) in the dye Coomassie brilliant blue G-250 when bound to arginine and hydrophobic amino acid residues. The increase in absorbency at 595 nm is proportional to the protein concentration in the sample. This assay was performed using the Biorad protein assay reagent.

[Protein] in µg / volume unit of sample tested = (OD_{595}x23) / Volume of sample tested.

3.2. Extraction of proteins.

Proteins from whole cells pellet were extracted as following. Logarithmically growing cells were centrifuged and washed with ice-cold water. Cell pellet was resuspended in one volume of extraction buffer (7.5% β-mercaptoethanol, 1.85 NaOH) and incubated 15 min on ice. Extracted proteins were precipitated 10 min on ice by addition of one volume of 55% TCA to the samples. TCA precipitate was centrifuged 10 min at 14000 rpm at 4°C. The precipitated proteins were resuspended in 100µl of HU buffer per unit OD_{600} of cells and incubated 10 min at 65°C. Equivalent to OD_{600} 0.2-0.5 of cells were analysed by SDS-PAGE and Western Blotting.

Alternatively, defined protein amounts from whole cell extracts after glassbeads extraction were solubilised in an excess of HU buffer (8 M urea, 5% SDS, 1.5% β-mercaptoethanol, 1mM EDTA, 200 mM Tris-HCl pH 6.8, 0.025% bromophenol blue) and incubated at 65°C 10min, cooled on ice and analysed by SDS-PAGE and Western Blotting.

3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE allows to separate proteins according to their molecular weight. Proteins and SDS are forming more or less negatively charged complexes depending on the length of the

proteins. These complexes are separated through the polyacrylamide gel according to the protein molecular weight. Molecular weights of the different proteins are estimated using protein markers of known molecular weight (NEB).

A discontinuous gel system was commonly used in this work, with a stacking gel (4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS) and a resolving gel (X% acrylamide depending on the protein's molecular weight to resolve, 375 mM Tris-HCl pH 8.8, 0.1% SDS). Gels were run 1 h at 120 V in 1X Tris-Glycine electrophoresis buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS).

For better resolution of small proteins 6 M urea was added in the stacking and resolving gel.

3.4. Western Blotting.

Separated proteins by SDS-PAGE are transferred from the gel to a solid support and immobilised. The membrane can be probed with specific antibody against the protein of interest allowing identification and quantification of a specific protein in complex mixtures.

In this work SDS-PAGE resolved proteins were transferred on PVDF membrane in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3 with HCl) using a semi-dry blot apparatus (Biorad) for 1 h at 24 V.

Immobilised proteins were stained with Ponceau S (0.5% Ponceau, 1% acetic acid).

3.5. Immunoblotting and detection.

Membranes were blocked minimum 1 h at room temperature or over-night at 4°C in blocking buffer (0.5% low-fat dry milk, 1X Tris Buffer Saline: 0.8% NaCl, 0.3% Tris, 0.02% KCl, pH 7.4). Membranes were incubated for 1 h at room temperature with the primary antibody diluted in blocking buffer, and washed 3 times 10 min at room temperature in 1X TBS. When required, membranes were incubated for 30-45 min at room temperature with a secondary antibody diluted in blocking buffer at and washed 3 times 10 min at room temperature in 1X TBS. Immunodetection was performed using a chemi-luminescence reaction depending on the peroxidase activity conjugated to the antibody. Substrate (POD - Roche) was added to the blot as suggested by the manufacturer. Signals were acquired and quantified using a Fuji LAS Reader 3000 (30 sec increment steps-standard binning), and AIDA (Raytest) respectively.

4. Additional biochemical methods.

4.1. Analysis of neo-synthesised rRNA.

Equivalent to 3 OD_{600} of cells were pelleted and resuspended in 1 ml of YPD including 20 µCi of 5', 6'[^{3}H] uracil (Amersham) for 15 min at 30°C. Total RNA was extracted (see materials & methods 2.2.1.), and the same amounts of radioactivity (cpm), as determined by scintillation counting, were loaded onto a denaturing agarose gel and then transferred onto a membrane. The membrane was exposed to a BAS-TR 2040 screen for 1 week. Radioactivity on membrane slices containing 25S and 18S rRNA was counted using a scintillation counter (Packard Tri-Carb 1600TR). Background activity was subtracted from the obtained values. The exact procedure was performed in duplicate for standardisation with the only exception being that the volumes of RNA loaded were the same. The resulting membranes were hybridised with a ^{32}P-labelled 25S probe. The amounts of labelled 25S rRNA in each strain were quantified using a PhosphorImager (Fuji). [^{3}H] activity was adjusted according to equal amounts of 25S rRNA on the gel and was expressed as a percentage of labelled wildtype RNA.

4.2. Purification of ribosomal subunits by ultra-centrifugation.

Logarithmically growing yeast cells (200 ml OD_{600} 0.4-0.6) are treated for 10 min with 20 mg cycloheximide, and cooled down on ice/ water for 15 min.

Cells are centrifuged and washed once with ice cold buffer A (20 mM HEPES pH 7.5, 100 mM KCl, 5 mM EGTA, 1 mM DTT, 100 µg/ml cycloheximide).

Cells are resuspended in ice cold buffer A, and broken with glass beads (0.75-1 mm - Roth) using a vibrax at full speed for 30 min at 4°C. Cell extracts are clarified by centrifugation (5 min 10000 rpm at 4°C). Five hundreds µg of proteins from the whole cell extracts are loaded on a 8-36% (w/v) sucrose gradient in buffer A without DTT and cycloheximide, and centrifuged for 3 h at 39000 rpm at 4°C.

Gradients were analysed using a BioLogic UV detection system (254 nm) connected to an FPLC-Pharmacia LKB-P500 (flow: 1 mL/min). Signals were recorded using the LP analysis software (Biorad).

4.3. Nuclear-cytoplasmic cell fractionation.

Twelve ml of cells at OD_{600} 1.2 were centrifuged and washed in 2 mM DTT and resuspended in recovery buffer (2% glucose, 1% peptone, 0.6% malt extract, 0.01% yeast extract, 12% mannitol, and 17.8 mM magnesium acetate) plus 1 mM DTT and 1 mg/ml zymolyase 100T (Seikagaku Corporation) for 30 min at 30°C with mild agitation.

Spheroplasted cells were washed once in recovery buffer and resuspended for 30 min at 30°C in recovery buffer and pulse labelled with 20 µCi of 5', 6' [^{3}H] uracil (Amersham) for 15 min at 30°C. Labelling reactions were stopped incubating the samples 5 min on ice/ water.

Nuclear and cytoplasmic fractions were then prepared. Cells were resuspended in 0.7 ml of buffer A (8% polyvinylpyrrolidon, 1 mM $MgCl_2$, 20 mM potassium phosphate buffer pH 6.5) and 0.03% (w/v) Triton-X 100, and directly broken by 22 strokes in a dounce tissue grinder (25-75 µm clearance, 1 ml volume, Wheaton). After addition of 0.7 ml of buffer A containing 0.6 M sucrose, the suspension was charged onto 4 ml of buffer A/0.45 M sucrose cushion and centrifuged for 10 min at 5000 rpm (4100 g) in a swing-out rotor at 4°C. In all, 500 µl of the turbid supernatant was recovered as the cytoplasmic fraction (≈35%); the pellet, corresponding to the nuclear fraction (100%), was resuspended in 150 µl of buffer A containing 0.45 M sucrose. Total RNA from the different fractions were extracted (materials & methods 2.2.1.). For normalisation, steady-state rRNA amounts in each fraction were determined by using a dot-blot experiment (see materials & methods 2.2.7.) with a ^{32}P–labelled probe hybridising with the 25S rRNA (see Table 4). Same amounts of "cold" RNA were then loaded on a denaturing agarose gel and transferred onto a membrane. Neo–synthesised rRNA were visualised either by phosphorimaging (BAS-TR2040 screen, Fuji; one week exposure) or by exposure of the blot to a BioMax MS film including a BioMax TranScreen (Kodak) at –80°C.

Steady-state amounts of pre-rRNA were analysed after hybridisation by using a DIG-labelled ITS1 probe (see materials & methods 2.2.8.2.).

4.4. RNA coimmunoprecipitation experiments.

Logarythmically growing cells were centrifuged and washed with ice-cold milli-Q water.

Cells were disrupted by vortexing at 4°C with glass beads (0.75-1 mm Roth) in buffer A200 containing 200 mM KCl, 20 mM Tris-HCl pH 8, 5 mM MgAc, 1 mM DTT, 1 mM PMSF,

2 mM benzamidine, 0.05 mM RVC, and 40 U/ml of RNasin (NEB). Extracts were clarified by two consecutive centrifugations at 14000 rpm for 5 and 10 minutes at 4°C. Triton X-100 was added to the supernatants to 0.2%. Five mg of whole cells extract were incubated with 50 µl of agarose anti-Flag M2 beads (Sigma) for 90 min at 4°C in A200T (200 mM KCl, 20 mM Tris pH 8, 5 mM MgAc, 1 mM DTT, 0.2% Triton X-100, 1 mM PMSF, 2 mM benzamidine, 0.05 mM ribonucleoside vanadyl complex).

Beads were washed 5 times with 2 ml of buffer A200T and 1 time with 10 ml of buffer A200T. RNA was extracted from beads and supernatant (see materials & methods 2.2.1.). Same volume-percent of extracted RNA from the corresponding beads and supernatant were loaded on denaturing agarose gels.

5. Cell biological method.

5.1. Fluorescence *in situ* hybridisation (FISH).

5.1.1. Cells preparation.

Cells growing in YPG were resuspended in pre-warmed YPD media (OD_{600} 0.5) for 2 h. Cells were fixed with 4% paraformaldehyde in YPD for 30 min at 30°C.Yeast cells were spherosplasted 30 min at 37°C in buffer B [20% (w/v) D (-) sorbitol, 100 mM phosphate buffer pH 7.5), supplemented with 2 mM ribonucleoside vanadyl complex, 0.2 mM PMSF, 2 mM benzamidine, 29 mM β-mercaptoethanol, and 50 µg/ml of zymolyase 100T (Seikagaku corporation)]. Spheroplasted cells were washed once with buffer B, and resuspended in ice cold buffer B. Spheroplast suspensions were dropped on poly-L-lysine coated cover slips, and incubated 30 min at 4°C. The cover slips were covered with cold 70% ethanol and incubated over-night at –20°C.

5.1.2. *In situ* hybridisation.

Cover slips are washed twice 5 min with 2X SSC and once in 2X SSC, 10% formamide at room temperature. Each cover slips are incubated in an humid chamber with 10 ng of Cy3-labelled-probe (see Cy3-ITS1 probe Table 4) solved in 2X SSC, 10% formamide, 10% dextran sulfate, 500 µg/ml tRNA (*E. coli*), 50 µg/ml BSA, 2 mM RVC between 3 h to over-night at 37°C.

Cover slips are washed (in the dark) twice 15 min in 2X SSC, 10% formamide at 37°C, followed by one washing step for 15 min 2X SSC, 0.1% Triton X-100, and two washing steps for 15 min in 1X SSC at room temperature.

Samples are incubated shortly in 1X TBS, and mounted on glass-slides in 5-8 µl in mowiol/ DAPI (1µg/ml).

5.1.3. Images acquisition and processing.

Images were captured with an AxioCam MR CCD camera coupled to a Zeiss Axiovert 200M microscope and processed with Zeiss Axiovision v 4.2 and Adobe Photoshop.

All the microscopy fields were acquired on the same day, with fixed parameters for each channel, and processed in batch with the same parameters.

6. Vectors and strains construction.

6.1. Vectors construction.

6.1.1. Cloning of the wildtype RPS genes.

Wildtype open reading frame coding for the ribosomal protein genes were PCR amplified from S288C yeast genomic DNA (Invitrogen) using the oligonucleotides described in Table 3. PCR fragments were cloned into vector Ycplac33 (URA3; CEN).

To allow correct expression of these genes, the ORF were cloned including their respective 5'UTR regulatory elements (Lascaris et al., 1999), and 300-500 bp after the stop codon were added as 3'UTR.

6.1.2. Cloning of conditionally expressed RPS genes.

Vectors containing conditional GAL-RPS genes were constructed as following.

The intron-less ORFs coding for the SSU r-proteins were PCR-amplified from +1/ATG to 3´UTR using the respective Ycplac33 vector containing the wildtype gene as template (see materials & methods 6.1.1.) and subsequently cloned into Ycplac111-Gal (LEU2; CEN) vector.

For the intron-containing ORF´s.

When possible, introns were bypassed by PCR using oligos homologous to exon1 and exon2, respectively, and amplified including the 3´UTR and cloned into Ycplac111-Gal vector.

RPS genes in which introns could not been bypassed by PCR were amplified using RT-PCR (see materials & methods 2.2.10.), and cloned into Ycplac111-Gal vector.

6.1.3. Cloning of conditionally expressed flag-tagged RPS genes.

Ribosomal proteins genes were cloned by PCR or sub-cloned from the vectors described in materials & methods 6.1.2. into pGAL-Nt-Flag or pGAL-Ct-Flag (LEU2; CEN) vectors for N or C terminal flag-tag respectively (see Table 1). Functionality of the vectors was tested on the basis of the complementation of loss of function of their respective r-protein gene.

6.1.4. Cloning of constitutively expressed flag-tagged RPS genes.

Ribosomal proteins genes were cloned by PCR or sub-cloned from the vectors described in materials & methods 6.1.3. into pRPS28Bprom-Nt-Flag or pRPS28Bprom-Ct-Flag (URA3; 2□) vectors for N or C terminal flag-tag respectively (see Table 1). Functionality of the vectors was tested on the basis of the complementation of loss of function of their respective r-protein gene.

6.2. Strains construction.

6.2.1. Shuffle strains construction.

In the case of duplicated r-proteins genes, a diploid strain was generated by mating two haploid yeast strains in which either one of the two ribosomal gene copies was replaced by a different selection marker (from KanMX4 to HIS3 or HIS3MX6 as described in Voth et al., 2003, see also Table 2). Diploid cells were transformed with a vector expressing a wildtype copy of the respective SSU r-protein (materials & methods 6.1.1.). Positive diploid transformants were selected, and sporulated. Tetrad analysis was performed, and haploid strains bearing deletions in both gene copies were selected ($G418^R$ and His^+).

Viability of the complete knockout strains was then tested on 5-FOA-plates (see materials & methods 1.2.1.4.).

6.2.2. Strains conditionally expressing the RPS genes.

Shuffle strains were transformed with their respective conditional RPS allele (materials & methods 6.1.2.). Positive transformants were selected, and platted onto 5-FOA-galactose plates in order to shuffle out the wildtype gene-containing vector. $5\text{-}FOA^R$ clones were selected indicating the functional complementation of the corresponding galactose dependent allele. Galactose dependent growth was then tested by platting out the $5\text{-}FOA^R$ clones on YP-glucose containing media.

6.2.3. Strains conditionally expressing the flag-tagged RPS genes.

Shuffle strains were transformed with their respective conditional flag-tagged RPS allele (materials & methods 6.1.2.). Positive transformants were selected, and platted onto

5-FOA-galactose containing media in order to shuffle out the wildtype gene-containing vector. 5-FOA^R clones were selected indicating the functional complementation of the corresponding galactose dependent flag-tagged allele. Galactose dependent growth was further tested by platting out the 5-FOA^R clones on YP-glucose containing media.

Table 1: List of plasmids used in this work.

ToP (Tschochner original Plasmide database entry)	**Plasmid name**	**Features**	**Comments**	**Origin**
	M4754		kanmx::HIS3 exchanging vector	(Voth et al., 2003)
□	pFA6a-GFP(S65T)-HIS3MX6	□	used as a kanmx::HIS3MX6 exchanging vector	(Longtine et al., 1998)
234	pGEMT-26AUAS-HIS3MX6-26ADS	□	Deletion vector RPS26A UAS-HIS3MX6-26A DS	Dr. Philipp Milkereit and Stephan Jellbauer
235	pGEMT-8BUAS-HIS3MX6-8BDS	□	Deletion vector RPS8B UAS-HIS3MX6-8B DS	Dr. Philipp Milkereit and Stephan Jellbauer
□	Ycplac33	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	□	(Gietz and Sugino, 1988)
287	Ycplac33-RPS0B	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 446-447 SacI-KpnI	This Study
288	Ycplac33-RPS1A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 448-449 SacI-KpnI	This Study
289	Ycplac33-RPS2	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 460-461 PstI-SacI	This Study
290	Ycplac33-RPS3	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 366-367 SacI-KpnI	This Study

ToP	Plasmid name	Features	Comments	Origin
291	Ycplac33-RPS5	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 368-369 SacI-KpnI	This Study
292	Ycplac33-RPS6A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 406-407 SacI-KpnI	This Study
294	Ycplac33-RPS8B	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 376-377 KpnI-HindIII	This Study
295	Ycplac33-RPS9A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 400-401 SacI-KpnI	This Study
296	Ycplac33-RPS10A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 452-453 SacI-HindIII	This Study
297	Ycplac33-RPS11A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 454-455 SacI-HindIII	This Study
298	Ycplac33-RPS13	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 370-371 SacI-KpnI	This Study
330	Ycplac33-RPS14A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 541-542 SacI-KpnI	This Study
299	Ycplac33-RPS16A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 402-403 SacI-KpnI	This Study
300	Ycplac33-RPS20	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 372-373 SacI-KpnI	This Study
303	Ycplac33-RPS23A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 458-459 KpnI-HindIII	This Study

ToP	Plasmid name	Features	Comments	Origin
304	Ycplac33-RPS24A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 374-375 SacI-HindIII	This Study
306	Ycplac33-RPS26A	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 462-463 PstI-KpnI	This Study
307	Ycplac33-RPS27B	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 444-445 SacI-KpnI	This Study
309	Ycplac33-RPS30B	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 394-395 SacI-KpnI	This Study
310	Ycplac33-RPS31	ORI (E.coli) f1;ARS1;CEN4 Amp URA3	Oligos 408-409 SacI-KpnI	This Study
230	Yplac111-GAL	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	BamHI/EcoRI Fragment containing GAL1/GAL10 promoter from Ycplac22GAL* clone into Ycplac111*	Dr. Philipp Milkereit
251	Ycplac111-pGAL-RPS0B	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	RT-PCR Oligos 481, 490, BamHI-PstI	This Study
254	Ycplac111-pGAL-RPS1A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 469-418 BamHI-PstI in ToP230	This Study
255	Ycplac111-pGAL-RPS2	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 491-488 BamHI-PstI in ToP230	This Study
257	Ycplac111-pGAL-RPS3	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 465-418 BamHI-PstI in ToP230	This Study

ToP	Plasmid name	Features	Comments	Origin
259	Ycplac111-pGAL-RPS5	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 466-418 BamHI-PstI in ToP230	This Study
261	Ycplac111-pGAL-RPS6A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 470-418 BamHI-PstI in ToP230	This Study
262	Ycplac111-pGAL-RPS8B	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 471-418 BamHI-PstI	This Study
264	Ycplac111-pGAL-RPS9A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 472-418 BamHI-PstI in ToP230	This Study
266	Ycplac111-pGAL-RPS10A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	RT-PCR with Oligos 493-482 BamHI-PstI in ToP230	This Study
268	Ycplac111-pGAL-RPS11A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 494-418 BamHI-PstI in ToP230	This Study
270	Ycplac111-pGAL-RPS13	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 464-418 BamHI-PstI in ToP230	This Study
338	Ycplac111-pGAL-RPS14A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 422-498 BamHI/PstI in ToP230	Gisela Pöll and Dr. Philipp Milkereit
272	Ycplac111-pGAL-RPS16A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 479-418 BamHI-PstI in ToP230	This Study
274	Ycplac111-pGAL-RPS20	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 467-418 BamHI-PstI in ToP230	This Study
276	Ycplac111-pGAL-RPS23	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 495-418 BamHI-PstI in ToP230	This Study

ToP	Plasmid name	Features	Comments	Origin
311	Ycplac111-pGAL-RPS24A	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 474-418 BamHI-PstI in ToP230	This Study
278	Ycplac111-pGAL-RPS26	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 496-489 BamHI-PstI in ToP230	This Study
280	Ycplac111-pGAL-RPS27	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 476-418 BamHI-PstI in ToP230	This Study
283	Ycplac111-pGAL-RPS30B	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 478-418 BamHI-PstI in ToP230	This Study
285	Ycplac111-pGAL-RPS31	ORI (E.coli) f1;ARS1;CEN4; Amp; LEU2	PCR with Oligos 468-418 BamHI-SphI in ToP230	This Study
	pfl38-pGAL			(Leger-Silvestre et al., 2004)
	pfl38-pGAL-RPS18B			(Leger-Silvestre et al., 2004)
□	pfl38-pGAL-RPS19	URA3	PCR from +1/ATG to stop codon in pFL38GAL	Prof. Dr. Gleizes
□	pfl38-pGAL-RPS7A	URA3	PCR from +1/ATG to stop codon in pFL38GAL	Prof. Dr. Gleizes
□	pfl38-pGAL-RPS28B	URA3	PCR from +1/ATG to stop codon in pFL38GAL	Prof. Dr. Gleizes
□	pDC-CRM1-T539C	pDC-CRM1-T539C; LEU2; CEN2	□	Neville and Rosbash, 1999
231	Ycplac111-pGAL-Flag	LEU2	□	Dr. Philipp Milkereit
252	Ycplac111-pGAL-Flag-RPS0B	RPS0B; LEU2	subclone BamHI-PstI ToP251 in in ToP231	This study

ToP	Plasmid name	Features	Comments	Origin
253	Ycplac111pGAL-Flag-RPS1A	RPS1A; LEU2	subclone BamHI-PstI ToP251 in ToP231	This study
256	Ycplac111-pGAL-Flag-RPS2	RPS2; LEU2	subclone BamHI-PstI ToP255 in ToP231	This study
258	Ycplac111-pGAL-Flag-RPS3	RPS3; LEU2	subclone BamHI-PstI ToP257 in ToP231	This study
260	Ycplac111-pGAL-Flag-RPS5	RPS5; LEU2	subclone BamHI-PstI ToP259 in ToP231	This study
414	Ycplac111-pGALRPS6A-Flag	RPS6A; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
265	Ycplac111-pGAL-Flag-RPS9A	RPS9A; LEU2	subclone BamHI-PstI ToP264 in ToP231	This study
267	Ycplac111-pGAL-Flag-RPS10A	RPS10A; LEU2	subclone BamHI-PstI ToP266 in ToP231	This study
269	Ycplac111-pGAL-Flag-RPS11A	RPS11A; LEU2	subclone BamHI-PstI ToP268 in ToP231	This study
271	Ycplac111-pGAL-Flag-RPS13	RPS13; LEU2	subclone BamHI-PstI ToP270 in ToP231	This study
339	Ycplac111-pGAL-Flag-RPS14	RPS14; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
237	Ycplac111-pGAL-Flag-RPS15	RPS15; LEU2	subclone BamHI-PstI ToP236 in ToP231	Dr. Philipp Milkereit
273	Ycplac111-pGAL-Flag-RPS16A	RPS16A; LEU2	subclone BamHI-PstI ToP272 in ToP231	This study
443	Ycplac111-GAL-Flag-RPS18	RPS18; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit

ToP	Plasmid name	Features	Comments	Origin
446	Ycplac111-GAL-RPS18-Flag	RPS18; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
341	Ycplac111-GAL-Flag-RPS19	RPS19; LEU2	subclone BamHI-PstI ToP251 in ToP231	Gisela Pöll and Dr. Philipp Milkereit
275	Ycplac111-pGAL-Flag-RPS20	RPS20; LEU2	subclone BamHI-PstI ToP272 in ToP231	This study
416	Ycplac111-pGAL-RPS24-Flag	RPS24; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
279	Ycplac111-GAL-RPS26A-Flag	RPS26A; LEU2	subclone BamHI-PstI ToP280 in ToP231	This study
281	Ycplac111-pGAL-Flag-RPS27B	RPS27B; LEU2	subclone BamHI-PstI ToP280 in ToP231	This study
417	Ycplac111-pGAL-RPS30B-Flag	RPS30B; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
418	Ycplac111-pGAL-RPS31-Flag	RPS31; LEU2	PCR BamHI-PstI in ToP231	Gisela Pöll and Dr. Philipp Milkereit
335	Ycplac111-GAL-Flag-NOC4	NOC4; LEU2; CEN4	□	Gisela Pöll and Dr. Philipp Milkereit
349	YEplac195-pRPS28Prom-Nterm FLAG	URA3; 2□	□	Dr. Philipp Milkereit
520	YEplac195-RPS28prom	URA3; 2□	□	Dr. Philipp Milkereit

ToP	Plasmid name	Features	Comments	Origin
421	pRPS28Bprom-Flag-RPS0B	RPS0B; URA3; 2	subclone BamHI-PstI ToP251 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
625	pRPS28Bprom-Flag-RPS1A	RPS1A; URA3; 2	subclone BamHI-PstI ToP611 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
624	pRPS28Bprom-Flag-RPS2	RPS2; URA3; 2	PCR BamHI-PstI in ToP349	Andreas Neueder
424	pRPS28Bprom-Flag-RPS3	RPS3; URA3; 2	subclone BamHI-PstI ToP257 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
351	pRPS28Bprom-Flag-RPS5	RPS5; URA3; 2	subclone BamHI-PstI ToP259 in ToP349	Dr. Philipp Milkereit
522	pRPS28Bprom-Flag-RPS6A	RPS6A; URA3; 2	subclone BamHI-PstI ToP414 in ToP487	This study
523	pRPS28Bprom-RPS9A-Flag	RPS9A; URA3; 2	subclone BamHI-PstI ToP415 in ToP487	This study
524	pRPS28Bprom-RPS10A-Flag	RPS10A; URA3; 2	subclone BamHI-PstI ToP390 in ToP487	This study
426	pRPS28Bprom-Flag-RPS11A	RPS11A; URA3; 2	subclone BamHI-PstI ToP268 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
427	pRPS28Bprom-Flag-RPS13	RPS13; URA3; 2	subclone BamHI-PstI ToP270 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
428	pRPS28Bprom-Flag-RPS14A	RPS14A; URA3; 2	subclone BamHI-PstI ToP339 in ToP349	Gisela Pöll and Dr. Philipp Milkereit

ToP	Plasmid name	Features	Comments	Origin
429	pRPS28Bprom-Flag-RPS15	RPS15; URA3; 2	subclone BamHI-PstI ToP236 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
622	pRPS28Bprom-Flag-RPS16A	RPS16A; URA3; 2	PCR BamHI-PstI in ToP349	Andreas Neueder
431	pRPS28Bprom-Flag-RPS17A	RPS17A; URA3; 2	subclone BamHI-PstI ToP340 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
432	pRPS28Bprom-Flag-RPS19A	RPS19A; URA3; 2	subclone BamHI-PstI ToP341 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
623	pRPS28Bprom-Flag-RPS20	RPS20; URA3; 2	PCR BamHI-PstI in ToP349	Andreas Neueder
487	pRPS28Bprom-RPS24A-Flag	RPS24A; URA3; 2	subclone BamHI-HindIII ToP416 in ToP520	This study
525	pRPS28Bprom-RPS26A-Flag	RPS26A; URA3; 2	subclone BamHI-PstI ToP391 in ToP487	This study
629	pRPS28Bprom-Flag-RPS27B	RPS27B; URA3; 2	subclone BamHI-PstI ToP616 in ToP349	Dr. Philipp Milkereit
434	pRPS28Bprom-Flag-RPS28B	RPS28B; URA3; 2	subclone BamHI-PstI ToP282 in ToP349	Gisela Pöll and Dr. Philipp Milkereit
766	pRPS28Bprom-Flag-RPS29a	RPS29A; URA3;2	subclone BamHI-PstI ToP751 in ToP349	Gisela Pöll
521	pRPS28Bprom-RPS31-Flag	RPS31; URA3; 2	subclone BamHI-HindIII ToP418 in ToP520	This study

Table 2: List of strains used in this work.

ToY (Tschochner original Yeast database entry)	**Strain name**	**Genotype**	**Vectors**	**Origin**
	BY4741-WT	Mat a;his3Δ1;leu2Δ0; met15Δ0; ura3Δ0		Euroscarf-Y00000
	BY4742-WT	Mat alpha;his3Δ1;leu2Δ0; lys2Δ0; ura3Δ0		Euroscarf-Y10000
141	RPS0A::HIS3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YGR214w:: HIS3;BY4741		This Study, derived from Euroscarf-Y04844
142	RPS6A::HIS3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YPL090c::HI S3;BY4741		This Study, derived from Euroscarf-Y02738
143	RPS9A::HIS3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YPL081w::H IS3;BY4741		This Study, derived from Euroscarf-Y02747
144	RPS10A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YOR293w:: HIS3;BY4741		This Study, derived from Euroscarf-Y01589
145	RPS11A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YDR025w:: HIS3;BY4741		This Study, derived from Euroscarf-Y03964
146	RPS23A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YGR118w:: HIS3;BY4741		This Study, derived from Euroscarf-Y04748
147	RPS25A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YGR027c::H IS3;BY4741		This Study, derived from Euroscarf-Y04657
148	RPS27A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YKL156w::H IS3;BY4741		This Study, derived from Euroscarf-Y05006
150	RPS30A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YLR287c-a::HIS3;BY4741		This Study, derived from Euroscarf-Y05197
157	RPS1A::HIS3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YLR441c::HI S3;BY4741		This Study, derived from Euroscarf-Y06045
159	RPS16A::HIS 3	his3Δ1;leu2Δ0;met15Δ 0;ura3Δ0;YMR143w:: HIS3;BY4741		This Study, derived from Euroscarf-Y00724

ToY	Strain name	Genotype	Vectors	Origin
160	RPS24A::HIS3	his3Δ1;leu2Δ0;met15Δ0;ura3Δ0;YER074w::HIS3;BY4741		This Study, derived from Euroscarf-Y00214
394	RPS14A::HIS3MX6	his3Δ1;leu2Δ0;met15Δ0;ura3Δ0;YCR031c::HIS3MX6; BY4741		This Study, derived from Euroscarf-Y05760
220	DeltaRPS8B	Mat alpha;his3Δ1;leu2Δ0; lys2Δ0; ura3Δ0; YER102w::HIS3MX6		This Study, derived from BY4742, KO with deletion vector ToP 235
221	DeltaRPS26A	Mat a;his3Δ1;leu2Δ0; met15Δ0; ura3Δ0; YGL189c::HIS3MX6		This Study, derived from BY4741, KO with deletion vector ToP 234
180	RPS0-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YLR048w::KanMX4;YGR214w::HIS3	Ycplac33-RPS0B	This Study *, derived from Euroscarf-Y12659 and ToY141
183	RPS1-Shuffle	his3Δ1;leu2Δ0;met15Δ0;LYS2;ura3Δ0;YML063w::KanMX4;YLR441c::HIS3	Ycplac33-RPS1A	This Study *, derived from Euroscarf-Y10507 and ToY157
186	RPS2-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; met15Δ0;lys2Δ0;YGL123w::KanMX4;	Ycplac33-RPS2	This Study *, derived from Euroscarf-Y24490
188	RPS9-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR189w::KanMX4;YPL081w::HIS3	Ycplac33-RPS9A	This Study *, derived from Euroscarf-Y16961 and ToY143
190	RPS13-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; MET15;LYS2;YDR064w::KanMX4	Ycplac33-RPS13	This Study *, derived from Euroscarf-Y23999
192	RPS20-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; MET15;LYS2;YHL015w::KanMX4	Ycplac33-RPS20	This Study *, derived from Euroscarf-Y20948
194	RPS30-Shuffle	his3Δ1;leu2Δ0;MET15;LYS2;ura3Δ0;YOR182c::KanMX4;YLR287c-a::HIS3	Ycplac33-RPS30B	This Study *, derived from Euroscarf-Y12438 and ToY150
195	RPS31-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; MET15;lys2Δ0;YLR167w::KanMX4;	Ycplac33-RPS31	This Study *, derived from Euroscarf-Y24116
198	RPS5-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; lys2Δ0;YJR123w::KanMX4;	Ycplac33-RPS5	This Study *, derived from Euroscarf-Y26926

ToY	Strain name	Genotype	Vectors	Origin
229	RPS6-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR 181c::KanMX4;YPL09 0c::HIS3	Ycplac33-RPS6A	This Study *, derived from Euroscarf-Y13321 and ToY142
233	RPS10-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YMR 230w::KanMX4;YOR2 93w::HIS3	Ycplac33-RPS10A	This Study *, derived from Euroscarf-Y10816 and ToY144
236	RPS16-Shuffle	his3Δ1;leu2Δ0; met15Δ0; LYS; ura3Δ0; YDL083c::KanMX4; YMR143w::HIS3	Ycplac33-RPS16A	This Study *, derived from Euroscarf-Y13780 and ToY159
240	RPS27-shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3∂0;YHR 021c::KanMX4;YL156 w::HIS3	Ycplac33-RPS27B	This Study *, derived from Euroscarf-Y10984 and ToY148
231	RPS8-shuffle	his3Δ1;leu2Δ0;lys2Δ0? ;ura3Δ0;met15Δ0?;YE R102w::HIS3MX6;YB L072c::KanMX4	YCplac33-RPS8A	This Study *, derived from Euroscarf-Y03098 and ToY220
239	RPS23-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; MET;ura3Δ0;YPR132 w::KanMX4;YGR118 w::HIS3	Ycplac33-RPS23A	This Study *, derived from Euroscarf-Y15547 and ToY146
265	RPS26-Shuffle	his3Δ1;leu2Δ0;lys2Δ0? ;ura3Δ0;met15Δ0?;YE R131w::KanMX4;YG L189c::HIS3MX6	Ycplac33-RPS26A	This Study *, derived from Euroscarf-Y16130 and ToY221
268	RPS3-Shuffle	his3Δ1;leu2Δ0;ura3Δ0; met15Δ0;lys2Δ0;YNL 178w::KanMX4	Ycplac33-RPS3	This Study*, derived from Euroscarf-Y22034
291	RPS11-Shuffle	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR 048w::KanMX4;YDR0 25w::HIS3	Ycplac33-RPS11A	This Study *, derived from Euroscarf-Y13185 and ToY145
363	RPS24-Shuffle	his3Δ1;leu2Δ0;lys2Δ0? ;met15Δ0?;ura3Δ0;YIL 069c::KanMX4;YER0 74w::HIS3	Ycplac33-RPS24	This Study *, derived from Euroscarf-Y11462 and ToY160
396	RPS14-Shuffle	his3Δ1;leu2Δ0;met15Δ 0;lys2Δ0;ura3Δ0;YCR 031c::HIS3MX6;YJL1 91w::KanMX4	Ycplac33-RPS14A	This Study *, derived from Euroscarf-Y17019 and ToY394

ToY	Strain name	Genotype	Vectors	Origin
	pGAL7	his3Δ1;leu2Δ0;ura3Δ0; YOR096w::KanMX4; YNL096c::KanMX4	pfl38-pGAL-RPS7A	Prof. Dr. Gleizes derived from Euroscarf-Y06475 and Euroscarf-Y17379
	pGAL28	his3Δ1;leu2Δ0;ura3Δ0; YOR167c::KanMX4;YLR274w::KanMX4	pfl38-pGAL-RPS28B	Prof. Dr. Gleizes derived from Euroscarf-Y02423 and Euroscarf-Y15173
224	pGAL-Flag-RPS15	his3Δ1;leu2Δ0;ura3Δ0; YOL040c::KanMX4;	Ycplac111-pGAL-Flag-RPS15	Dr. Philipp Milkereit derived from pGAL-RPS15 (Leger-Silvestre et al 2004)
256	pGAL-RPS0	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YLR048w::KanMX4;YGR214w::HIS3	Ycplac111pGAL-RPS0B	This Study, derived from ToY 180
257	pGAL-RPS1	his3Δ1;leu2Δ0;met15Δ0;LYS2;ura3Δ0;YML063w::KanMX4;YLR441c::HIS3	Ycplac111pGAL-RPS1A	This Study, derived from ToY 183
258	pGAL-RPS6	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR181c::KanMX4;YPL090c::HIS3	Ycplac111-pGALRPS6A	This Study, derived from ToY 229
259	pGAL-RPS9	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR189w::KanMX4;YPL081w::HIS3	Ycplac111-pGALRPS9A	This Study, derived from ToY 188
260	pGAL-RPS10	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YMR230w::KanMX4;YOR293w::HIS3	Ycplac111-pGALRPS10A	This Study, derived from ToY 233
261	pGAL-RPS13	his3Δ1;leu2Δ0;ura3Δ0; MET15;LYS2;YDR064w::KanMX4	Ycplac111-pGALRPS13	This Study, derived from ToY 190
262	pGAL-RPS20	his3Δ1;leu2Δ0;ura3Δ0; met15Δ0;LYS2;YHL015w::KanMX4	Ycplac111-pGALRPS20	This Study, derived from ToY 192
263	pGAL-RPS30	his3Δ1;leu2Δ0;MET15;LYS2;ura3Δ0;YOR182c::KanMX4;YLR287c-a::HIS3	Ycplac111-pGALRPS30B	This Study, derived from ToY 194

ToY	Strain name	Genotype	Vectors	Origin
286	pGAL-RPS2	his3Δ1;leu2Δ0;ura3Δ0; met15Δ0;lys2Δ0;YGL 123w::KanMX4;	Ycplac111-pGAL-RPS2	This Study, derived from ToY 186
287	pGAL-RPS8	his3Δ1;leu2Δ0;lys2Δ0? ;ura3Δ0;met15Δ0?;YE R102w::HIS3MX6;YB L072c::KanMX4	YCplac111-pGAL-RPS8A	This Study, derived from ToY 231
288	pGAL-RPS23	his3Δ1;leu2Δ0;LYS;m et15Δ0;ura3Δ0;YPR13 2w::KanMX4;YGR118 w::HIS3	Ycplc111-pGAL-RPS23A	This Study, derived from ToY 239
318	pGAL-RPS16	his3Δ1;leu2Δ0;met15Δ 0;LYS;ura3Δ0;YDL08 3c::KanMX4;YMR143 w::HIS3	Ycplac111-pGAL-RPS16A	This Study, derived from ToY 236
323	pGAL-RPS5	his3Δ1;leu2Δ0;ura3Δ0; lys2Δ0 ;YJR123w::KanMX4;	Ycplac111-pGAL-RPS5	This Study, derived from ToY 198
325	pGAL-RPS11	his3Δ1;leu2Δ0;lys2Δ0; met15Δ0;ura3Δ0;YBR 048w::KanMX4;YDR0 25w::HIS3	Ycplac111-pGAL-RPS11A	This Study, derived from ToY 291
326	pGAL-RPS27	his3Δ1;leu2Δ0;LYS;m et15Δ0;ura3Δ0;YHR02 1c::KanMX4;YL156w: :HIS3	Ycplac111-pGAL-RPS27B	This Study, derived from ToY 240
327	pGAL-RPS3	his3Δ1;leu2Δ0;ura3Δ0; met15Δ0?;lys2Δ0?;YN L178w::KanMX4	Ycplac111-pGAL-RPS3	This Study, derived from ToY 268
336	pGAL-RPS31	his3Δ1;leu2Δ0;ura3Δ0; MET15;lys2Δ0;YLR16 7w::KanMX4;	Ycplac111-pGAL-RPS31	This Study, derived from ToY 195
340	pGAL-RPS26	his3Δ1;leu2Δ0;lys2Δ0? ;ura3Δ0;met15Δ0?;YE R131w::KanMX4;YG L189c::HIS3MX6	Ycplac111-GAL-RPS26A	This Study, derived from ToY 265
	pGAL-RPS18		pfl38-pGAL-RPS18	(Leger-Silvestre et al., 2004)
	pGAL-RPS19	his3Δ1;leu2Δ0;ura3Δ0; YOL121c::KanMX4;Y NL302c::KanMX4	pfl38-pGAL-RPS19	(Leger-Silvestre et al., 2005)

ToY	Strain name	Genotype	Vectors	Origin
344	ΔRPS25A/ΔRPS25B	his3Δ1;leu2Δ0;lys2Δ0?;met15Δ0?;ura3Δ0;YLR333c::KanMX4;YGR027c::HIS3		This Study
365	pGAL-RPS24	his3Δ1;leu2Δ0;lys2Δ0?;met15Δ0?;ura3Δ0;YIL069c::KanMX4;YER074w::HIS3	Ycplac111-pGAL-RPS24	This Study, derived from ToY 363
399	pGAL-RPS14	his3Δ1;leu2Δ0;met15Δ0;lys2Δ0;ura3Δ0;YCR031c::HIS3MX6;YJL191w::KanMX4	Ycplac111-pGAL-RPS14	This Study*, derived from ToY 396
	ΔRPS12	Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR369c::kanMX4		Euroscarf-Y01666
405	pGAL-Flag-NOC4	his3Δ; leu2Δ0; lys2Δ0; ura3Δ0; YPR144c::kanMX4	Ycplac111-pGAL-Flag-NOC4	Dr. Philipp Milkereit derived from ToY30 aka NOC4-Shuffle (Milkereit et al., 2003b)
271	pGAL-Flag-RPS0	his3Δ1;leu2Δ0;lys2Δ0;met15Δ0;ura3Δ0;YLR048w::KanMX4;YGR214w::HIS3	Ycplac111-pGAL-Flag-RPS0B	This Study, derived from ToY 180
316	pGAL-Flag-RPS1	his3Δ1;leu2Δ0;met15Δ0;LYS2;ura3Δ0;YML063w::KanMX4;YLR441c::HIS3	Ycplac111pGAL-Flag-RPS1A	This Study, derived from ToY 183
508	pGAL-RPS6-Flag	his3Δ1;leu2Δ0;lys2Δ0;met15Δ0;ura3Δ0;YBR181c::KanMX4;YPL090c::HIS3	Ycplac111-pGALRPS6A-Flag	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 229
289	pGAL-Flag-RPS9	his3Δ1;leu2Δ0;lys2Δ0;met15Δ0;ura3Δ0;YBR189w::KanMX4;YPL081w::HIS3	Ycplac111-pGAL-Flag-RPS9A	This Study, derived from ToY 188
290	pGAL-Flag-RPS10	his3Δ1;leu2Δ0;lys2Δ0;met15Δ0;ura3Δ0;YMR230w::KanMX4;YOR293w::HIS3	Ycplac111-pGAL-Flag-RPS10A	This Study, derived from ToY 233
272	pGAL-Flag-RPS13	his3Δ1;leu2Δ0;ura3Δ0;MET15;LYS2;YDR064w::KanMX4	Ycplac111-pGAL-Flag-RPS13	This Study, derived from ToY 190

ToY	Strain name	Genotype	Vectors	Origin
320	pGAL-Flag-RPS20	his3Δ1;leu2Δ0;ura3Δ0;met15Δ0;LYS2;YHL015w::KanMX4	Ycplac111-pGAL-Flag-RPS20	This Study, derived from ToY 192
511	pGAL-RPS30-Flag	his3Δ1;leu2Δ0;MET15;LYS2;ura3Δ0;YOR182c::KanMX4;YLR287c-a::HIS3	Ycplac111-pGAL-RPS30B-Flag	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 194
335	pGAL-Flag-RPS2	his3Δ1;leu2Δ0;ura3Δ0;met15Δ0;lys2Δ0;YGL123w::KanMX4;	Ycplac111-pGAL-Flag-RPS2	This Study, derived from ToY 186
319	pGAL-Flag-RP16	his3Δ1;leu2Δ0;met15Δ0;LYS;ura3Δ0;YDL083c::KanMX4;YMR143w::HIS3	Ycplac111-pGAL-Flag-RPS16A	This Study, derived from ToY 236
324	pGAL-Flag-RPS5	his3Δ1;leu2Δ0;ura3Δ0;lys2Δ0;YJR123w::KanMX4;	Ycplac111-pGAL-Flag-RPS5	This Study, derived from ToY 198
322	pGAL-Flag-RPS11	his3Δ1;leu2Δ0;lys2Δ0;met15Δ0;ura3Δ0;YBR048w::KanMX4;YDR025w::HIS3	Ycplac111-pGAL-Flag-RPS11A	This Study, derived from ToY 291
321	pGAL-Flag-RPS27	his3Δ1;leu2Δ0;LYS;met15Δ0;ura3Δ0;YHR021c::KanMX4;YL156w::HIS3	Ycplac111-pGAL-Flag-RPS27B	This Study, derived from ToY 241
317	pGAL-Flag-RPS3	his3Δ1;leu2Δ0;ura3Δ0;met15Δ0?;lys2Δ0?;YNL178w::KanMX4	Ycplac111-pGAL-Flag-RPS3	This Study, derived from ToY 268
512	pGAL-RPS31-Flag	his3Δ1;leu2Δ0;ura3Δ0;MET15;lys2Δ0;YLR167w::KanMX4;	Ycplac111-pGAL-RPS31-Flag	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 195
485	pGAL-RPS26-Flag	his3Δ1;leu2Δ0;lys2Δ0?;ura3Δ0;met15Δ0?;YER131w::KanMX4;YGL189c::HIS3MX6	Ycplac111-GAL-RPS26A-Flag	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 265
541	pGAL-Flag-RPS18		Ycplac111-GAL-Flag-RPS18	Dr. Philipp Milkereit derived from pGAL-RPS18 (Leger-Silvestre et al., 2004)
542	pGAL-RPS18-Flag		Ycplac111-GAL-RPS18-Flag	Dr. Philipp Milkereit derived from pGAL-RPS18 (Leger-Silvestre et al., 2004)

ToY	Strain name	Genotype	Vectors	Origin
367	pGAL-Flag-RPS19	his3Δ1;leu2Δ0;ura3Δ0; YOL121c::KanMX4;YNL302c::KanMX4	Ycplac111-GAL-Flag-RPS19	This Study, derived from pGAL-RPS19 (Leger-Silvestre et al., 2005)
510	pGAL-RPS24-Flag	his3Δ1;leu2Δ0;lys2Δ0?;met15Δ0?;ura3Δ0;YIL069c::KanMX4;YER074w::HIS3	Ycplac111-pGAL-RPS24-Flag	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 363
400	pGAL-Flag-RPS14	his3Δ1;leu2Δ0;met15Δ0;lys2Δ0;ura3Δ0;YCR031c::HIS3MX6;YJL191w::KanMX4	Ycplac111-pGAL-Flag-RPS14	Gisela Pöll and Dr. Philipp Milkereit, derived from ToY 396
89	pGAL-RPS15	his3Δ; leu2Δ0; ura3Δ0; YOL040c::KanMX4	pFL36-GAL-RPS15	This study derived from pFL38-GAL-RPS15 (Leger-Silvestre et al., 2004)
376	Crm1-T539C	Mat alpha; leu2Δ0; his3Δ; ura3Δ0; ade2::hisG; can1::hisG; trp1::hisG; YGR218w::HygB	pDC-CRM1-T539C	Dr. Holger Kühn, derived from Euroscarf Y24848

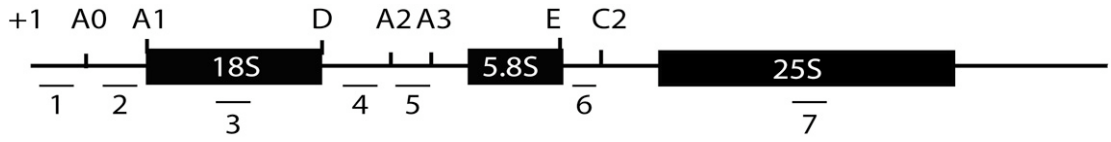
*** in common work with Gisela Pöll.**

Table 3: List of oligos used for PCR in this work

ToO	position	restriction enzyme	Gene name / comments
□	□ □	□	□
366	□ -300	□ SacI	RPS3
367	□ +300 stop	□ KpnI	RPS3
368	□ -600	□ SacI	RPS5
369	□ +300 stop	□ KpnI	RPS5
370	□ -250	□ SacI	RPS13
371	□ +300 stop	□ KpnI	RPS13
372	□ -300	□ SacI	RPS20
373	□ +300 stop	□ KpnI	RPS20
374	□ -400	□ SacI	RPS24A
375	□ +300 stop	□ HindIII	RPS24A
376	□ -800	□ KpnI	RPS8B
377	□ +300 stop	□ HindIII	RPS8B
394	□ -300	□ Sac I	RPS30B
395	□ +300 stop	□ Kpn I	RPS30B
400	□ -300	□ Sac I	RPS9A
401	□ +300 stop	□ Kpn I	RPS9A
402	□ -500	□ Sac I	RPS16A
403	□ +500 stop	□ Kpn I	RPS16A
406	□ -500	□ Sac I	RPS6A
407	□ +300 stop	□ Kpn I	RPS6A
408	□ -300	□ Sac I	RPS31
409	□ +300 stop	□ Kpn I	RPS31
444	□ -500	□ SacI	RPS27B
445	□ +500 stop	□ KpnI	RPS27B
446	□ -500	□ SacI	RPS0B
447	□ +300 stop	□ KpnI	RPS0B
448	□ -300	□ SacI	RPS1A
449	□ +300 stop	□ KpnI	RPS1A
452	□ -600	□ SacI	RPS10A
453	□ +300 stop	□ HindIII	RPS10A
454	□ -400	□ SacI	RPS11A
455	□ +300 stop	□ HindIII	RPS11A
458	□ -400	□ KpnI	RPS23A
459	□ +300 stop	□ HindIII	RPS23A
460	□ -500	□ PstI	RPS2
461	□ +300 stop	□ SacI	RPS2

ToO	position	restriction enzyme	Gene name / comments
462	-800	PstI	RPS26A
463	+300 stop	KpnI	RPS26A
541	-300	SacI	RPS14A
542	+300 stop	KpnI	RPS14A
□	□	□	□
418	□	□	YCplac-ORI-HindtoEco
422	+300 stop	PstI	RPS14A Rev
464	ATG/+1	BamHI	RPS13 Exonbypass GAL F
465	ATG/+1	BamHI	RPS3 GAL F
466	ATG/+1	BamHI	RPS5 GAL F
467	ATG/+1	BamHI	RPS20 GAL F
468	ATG/+1	BamHI	RPS31 GAL F
469	ATG/+1	BamHI	RPS1A GAL F
470	ATG/+1	BamHI	RPS6A Exonbypass GAL F
471	ATG/+1	BamHI	RPS8B GAL F
472	ATG/+1	BamHI	RPS9A Exonbypass GAL F
476	ATG/+1	BamHI	RPS27B Exonbypass GAL F
478	ATG/+1	BamHI	RPS30B Exonbypass GAL F
479	ATG/+1	BamHI	RPS16A Exonbypass GAL F
481	stop	PstI	RT GalRPS0B Rev
482	stop	PstI	RT GalRPS10A Rev
488	+300 stop	PstI	GalRPS2 Rev
490	ATG/+1	BamHI	RT GalRPS0B F
491	ATG/+1	BamHI	GalRPS2 F
493	ATG/+1	BamHI	RT GalRPS10A F
494	ATG/+1	BamHI	RT GalRPS11A F
495	ATG/+1	BamHI	RT GalRPS23A F
496	ATG/+1	BamHI	GalRPS26A F
498	ATG/+1	BamHI	RPS14A F

Table 4: List of probes used in this work for rRNA detection.



Database access	Probe	Sequence	Target Region	rRNA species detected
204	1	5´-GGT CTC TCT GCT GCC GG-3´	1-A0	35S and 23S
119	2	5´-GAA GA GCA ACA AGC AGT-3´	A0-A1	35S, 32S, 23S and 22S
205	3	5´-CAT GGC TTA ATC TTT GAG AC-3´	18S rRNA	35S, 32S, 23S, 22S, 21S, 20S and 18S
□	4-DIG	*in vitro* trancription D-A2	D-A2	35S, 32S, 23S, 22S, 21S and 20S
207	5	5´-TGT TAC CTC TGG GCC C-3´	A2-A3	35S, 32S, 23S, 22S, and 21S
210	6	5´-GGC CAG CAA TTT CAA GTT-3´	E-C2	35S, 32S, 27S(all), and 7S
212	7	5´-CTC CGC TTA TTG ATA TGC-3´	25S rRNA	35S, 32S, 27S(all), and 25S
□	□	□	□	□
□	RPS0B mRNA	5´-CT GCA TTT TCT TCA GCC CA-3´	Exon 2 1083-1101	□
□	RPS30A/B mRNA	5´-TGG ACC TGG GTT CAT TCT TCT-3´	Exon2 30A 590-610 / 30B 571-591	□
□	RPS31 mRNA	5´-CAA GAA AAC ACC AGC ACC ACA-3´	376-396	□
□	Cy3-ITS1	5´-TT*GCACAGAAATCTCT*CACCGT TTGGAAT*AGCAAGAAAGAAACT*T ACAAGCT*T-3	D-A2	□

T* represents amino-modified deoxythymidine conjugated to Cy3.

REFERENCES

Adam, S. A., Nakagawa, T., Swanson, M. S., Woodruff, T. K., and Dreyfuss, G. (1986). mRNA polyadenylate-binding protein: gene isolation and sequencing and identification of a ribonucleoprotein consensus sequence. Mol Cell Biol *6*, 2932-2943.

Agalarov, S. C., Selivanova, O. M., Zheleznyakova, E. N., Zheleznaya, L. A., Matvienko, N. I., and Spirin, A. S. (1999). Independent in vitro assembly of all three major morphological parts of the 30S ribosomal subunit of Thermus thermophilus. Eur J Biochem *266*, 533-537.

Agalarov, S. C., Zheleznyakova, E. N., Selivanova, O. M., Zheleznaya, L. A., Matvienko, N. I., Vasiliev, V. D., and Spirin, A. S. (1998). In vitro assembly of a ribonucleoprotein particle corresponding to the platform domain of the 30S ribosomal subunit. Proc Natl Acad Sci U S A *95*, 999-1003.

Agrawal, R. K., Spahn, C. M., Penczek, P., Grassucci, R. A., Nierhaus, K. H., and Frank, J. (2000). Visualization of tRNA movements on the Escherichia coli 70S ribosome during the elongation cycle. J Cell Biol *150*, 447-460.

Aitchison, J. D., and Rout, M. P. (2000). The road to ribosomes. Filling potholes in the export pathway. J Cell Biol *151*, F23-26.

Alix, J. H., and Nierhaus, K. H. (2003). DnaK-facilitated ribosome assembly in Escherichia coli revisited. Rna *9*, 787-793.

Alksne, L. E., Anthony, R. A., Liebman, S. W., and Warner, J. R. (1993). An accuracy center in the ribosome conserved over 2 billion years. Proc Natl Acad Sci U S A *90*, 9538-9541.

Allmang, C., Henry, Y., Wood, H., Morrissey, J. P., Petfalski, E., and Tollervey, D. (1996). Recognition of cleavage site A(2) in the yeast pre-rRNA. Rna *2*, 51-62.

Allmang, C., and Tollervey, D. (1998). The role of the 3' external transcribed spacer in yeast pre-rRNA processing. J Mol Biol *278*, 67-78.

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. Genes Dev *6*, 1173-1189.

Andersen, N. M., and Douthwaite, S. (2006). YebU is a m5C methyltransferase specific for 16 S rRNA nucleotide 1407. J Mol Biol *359*, 777-786.

Anderson, R. M., Kwon, M., and Strobel, S. A. (2007). Toward Ribosomal RNA Catalytic Activity in the Absence of Protein. J Mol Evol.

Arnold, R. J., and Reilly, J. P. (1999). Observation of Escherichia coli ribosomal proteins and their posttranslational modifications by mass spectrometry. Anal Biochem *269*, 105-112.

Arnold, R. J., and Reilly, J. P. (2002). Analysis of methylation and acetylation in E. coli ribosomal proteins. Methods Mol Biol *194*, 205-210.

Atzorn, V., Fragapane, P., and Kiss, T. (2004). U17/snR30 is a ubiquitous snoRNA with two conserved sequence motifs essential for 18S rRNA production. Mol Cell Biol *24*, 1769-1778.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol *2*, 2006 0008.

Bachellerie, J. P., Cavaille, J., and Huttenhofer, A. (2002). The expanding snoRNA world. Biochimie *84*, 775-790.

Badis, G., Fromont-Racine, M., and Jacquier, A. (2003). A snoRNA that guides the two most conserved pseudouridine modifications within rRNA confers a growth advantage in yeast. Rna *9*, 771-779.

Badis, G., Saveanu, C., Fromont-Racine, M., and Jacquier, A. (2004). Targeted mRNA degradation by deadenylation-independent decapping. Mol Cell *15*, 5-15.

Baliga, B. S., Pronczuk, A. W., and Munro, H. N. (1969). Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver. J Biol Chem *244*, 4480-4489.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science *289*, 905-920.

Bar-Nun, S., Shneyour, Y., and Beckmann, J. S. (1983). G-418, an elongation inhibitor of 80 S ribosomes. Biochim Biophys Acta *741*, 123-127.

Bataille, N., Helser, T., and Fried, H. M. (1990). Cytoplasmic transport of ribosomal subunits microinjected into the Xenopus laevis oocyte nucleus: a generalized, facilitated process. J Cell Biol *111*, 1571-1582.

Baudin-Baillieu, A., Tollervey, D., Cullin, C., and Lacroute, F. (1997). Functional analysis of Rrp7p, an essential yeast protein involved in pre-rRNA processing and ribosome assembly. Mol Cell Biol *17*, 5023-5032.

Bayliss, R., Littlewood, T., and Stewart, M. (2000). Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking. Cell *102*, 99-108.

Bayliss, R., Ribbeck, K., Akin, D., Kent, H. M., Feldherr, C. M., Gorlich, D., and Stewart, M. (1999). Interaction between NTF2 and xFxFG-containing nucleoporins is required to mediate nuclear import of RanGDP. J Mol Biol *293*, 579-593.

Beckmann, R., Spahn, C. M., Eswar, N., Helmers, J., Penczek, P. A., Sali, A., Frank, J., and Blobel, G. (2001). Architecture of the protein-conducting channel associated with the translating 80S ribosome. Cell *107*, 361-372.

Bednenko, J., Cingolani, G., and Gerace, L. (2003). Importin beta contains a COOH-terminal nucleoporin binding region important for nuclear transport. J Cell Biol *162*, 391-401.

Beelman, C. A., and Parker, R. (1995). Degradation of mRNA in eukaryotes. Cell *81*, 179-183.

Beltrame, M., Henry, Y., and Tollervey, D. (1994). Mutational analysis of an essential binding site for the U3 snoRNA in the 5' external transcribed spacer of yeast pre-rRNA. Nucleic Acids Res *22*, 5139-5147.

Beltrame, M., and Tollervey, D. (1992). Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. Embo J *11*, 1531-1542.

Beltrame, M., and Tollervey, D. (1995). Base pairing between U3 and the pre-ribosomal RNA is required for 18S rRNA synthesis. Embo J *14*, 4350-4356.

Bernstein, K. A., Gallagher, J. E., Mitchell, B. M., Granneman, S., and Baserga, S. J. (2004). The small-subunit processome is a ribosome assembly intermediate. Eukaryot Cell *3*, 1619-1626.

Bernstein, K. A., Granneman, S., Lee, A. V., Manickam, S., and Baserga, S. J. (2006). Comprehensive mutational analysis of yeast DEXD/H box RNA helicases involved in large ribosomal subunit biogenesis. Mol Cell Biol *26*, 1195-1208.

Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., et al. (1997). The complete genome sequence of Escherichia coli K-12. Science *277*, 1453-1474.

Boeke, J. D., LaCroute, F., and Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet *197*, 345-346.

Brachet, J. (1933). Synthesis of thymonucleic acid in sea-urchin egg. Arch Biol, 519-576.

Brachet, J. (1942). La localisation des acides pentosennucléiques dans les tissus animaux et les oeufs d´Amphibiens en voie de développement. Arch Biol, 207-257.

Bradatsch, B., Katahira, J., Kowalinski, E., Bange, G., Yao, W., Sekimoto, T., Baumgartel, V., Boese, G., Bassler, J., Wild, K., et al. (2007). Arx1 Functions as an Unorthodox Nuclear Export Receptor for the 60S Preribosomal Subunit. Mol Cell *27*, 767-779.

Bram, R. J., Young, R. A., and Steitz, J. A. (1980). The ribonuclease III site flanking 23S sequences in the 30S ribosomal precursor RNA of E. coli. Cell *19*, 393-401.

Brenner, S., Jacob, F., and Meselson, M. (1961). An Unstable Intermediate Carrying Information from Genes to Ribosomes for Protein Synthesis. Nature *190*, 576-581.

Briggs, M. W., Burkard, K. T., and Butler, J. S. (1998). Rrp6p, the yeast homologue of the human PM-Scl 100-kDa autoantigen, is essential for efficient 5.8 S rRNA 3' end formation. J Biol Chem *273*, 13255-13263.

Brodersen, D. E., Clemons, W. M., Jr., Carter, A. P., Wimberly, B. T., and Ramakrishnan, V. (2002). Crystal structure of the 30 S ribosomal subunit from Thermus thermophilus: structure of the proteins and their interactions with 16 S RNA. J Mol Biol *316*, 725-768.

Brodersen, D. E., and Nissen, P. (2005). The social life of ribosomal proteins. Febs J *272*, 2098-2108.

Brown, D. D., and Gurdon, J. B. (1964). Absence of Ribosomal Rna Synthesis in the Anucleolate Mutant of Xenopus Laevis. Proc Natl Acad Sci U S A *51*, 139-146.

Bryk, M., Banerjee, M., Murphy, M., Knudsen, K. E., Garfinkel, D. J., and Curcio, M. J. (1997). Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. Genes Dev *11*, 255-269.

Bubunenko, M., Baker, T., and Court, D. L. (2007). Essentiality of ribosomal and transcription antitermination proteins analyzed by systematic gene replacement in Escherichia coli. J Bacteriol *189*, 2844-2853.

Bubunenko, M., Korepanov, A., Court, D. L., Jagannathan, I., Dickinson, D., Chaudhuri, B. R., Garber, M. B., and Culver, G. M. (2006). 30S ribosomal subunits can be assembled in vivo without primary binding ribosomal protein S15. Rna *12*, 1229-1239.

Buchhaupt, M., Meyer, B., Kotter, P., and Entian, K. D. (2006). Genetic evidence for 18S rRNA binding and an Rps19p assembly function of yeast nucleolar protein Nep1p. Mol Genet Genomics *276*, 273-284.

Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., et al. (1996). Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii. Science *273*, 1058-1073.

Burman, W. J., Breese, P. E., Murray, B. E., Singh, K. V., Batal, H. A., MacKenzie, T. D., Ogle, J. W., Wilson, M. L., Reves, R. R., and Mehler, P. S. (2003). Conventional and molecular epidemiology of trimethoprim-sulfamethoxazole resistance among urinary Escherichia coli isolates. Am J Med *115*, 358-364.

Carpousis, A. J. (2002). The Escherichia coli RNA degradosome: structure, function and relationship in other ribonucleolytic multienzyme complexes. Biochem Soc Trans *30*, 150-155.

Cate, J. H., Yusupov, M. M., Yusupova, G. Z., Earnest, T. N., and Noller, H. F. (1999). X-ray crystal structures of 70S ribosome functional complexes. Science *285*, 2095-2104.

Cavaille, J., Nicoloso, M., and Bachellerie, J. P. (1996). Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. Nature *383*, 732-735.

Cech, T. R. (2000). Structural biology. The ribosome is a ribozyme. Science *289*, 878-879.

Chamberlain, J. R., Lee, Y., Lane, W. S., and Engelke, D. R. (1998). Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. Genes Dev *12*, 1678-1690.

Chandler, D. (2005). Interfaces and the driving force of hydrophobic assembly. Nature *437*, 640-647.

Chanfreau, G., Buckle, M., and Jacquier, A. (2000). Recognition of a conserved class of RNA tetraloops by Saccharomyces cerevisiae RNase III. Proc Natl Acad Sci U S A *97*, 3142-3147.

Chang, B., Halgamuge, S., and Tang, S. L. (2006). Analysis of SD sequences in completed microbial genomes: non-SD-led genes are as common as SD-led genes. Gene *373*, 90-99.

Changchien, L. M., and Craven, G. R. (1978). Studies on the role of amino acid residues 31 through 46 of ribosomal protein S4 in the mechanism of 30 S ribosome assembly. J Mol Biol *125*, 43-56.

Changchien, L. M., and Craven, G. R. (1979). Analysis of protein--protein relationships in 30S ribosome assembly intermediates using protection from proteolytic digestion. Biochemistry *18*, 1275-1281.

Chao, F. C. (1957). Dissociation of macromolecular ribonucleoprotein of yeast. Arch Biochem Biophys *70*, 426-431.

Chao, F. C. (1961). The isolation and characterization of ribonucleic acid from yeast macromolecular ribonucleoprotein. Biochim Biophys Acta *53*, 64-69.

Chao, F. C., and Schachman, H. K. (1956). The isolation and characterization of a macro-molecular ribonucleoprotein from yeast. Arch Biochem Biophys *61*, 220-230.

Chelladurai, B. S., Li, H., and Nicholson, A. W. (1991). A conserved sequence element in ribonuclease III processing signals is not required for accurate in vitro enzymatic cleavage. Nucleic Acids Res *19*, 1759-1766.

Chen, D., Zhang, Z., Li, M., Wang, W., Li, Y., Rayburn, E. R., Hill, D. L., Wang, H., and Zhang, R. (2007). Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene *26*, 5029-5037.

Cheng, T. H., Chang, C. R., Joy, P., Yablok, S., and Gartenberg, M. R. (2000). Controlling gene expression in yeast by inducible site-specific recombination. Nucleic Acids Res *28*, E108.

Choesmel, V., Bacqueville, D., Rouquette, J., Noaillac-Depeyre, J., Fribourg, S., Cretien, A., Leblanc, T., Tchernia, G., Dacosta, L., and Gleizes, P. E. (2006). Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood.

Chooi, W. Y., and Leiby, K. R. (1981). An electron microscopic method for localization of ribosomal proteins during transcription of ribosomal DNA: a method for studying protein assembly. Proc Natl Acad Sci U S A *78*, 4823-4827.

Christiansen, J. (1988). The 9S RNA precursor of Escherichia coli 5S RNA has three structural domains: implications for processing. Nucleic Acids Res *16*, 7457-7476.

Chu, S., Archer, R. H., Zengel, J. M., and Lindahl, L. (1994). The RNA of RNase MRP is required for normal processing of ribosomal RNA. Proc Natl Acad Sci U S A *91*, 659-663.

Ciammaruconi, A., and Londei, P. (2001). In vitro processing of the 16S rRNA of the thermophilic archaeon Sulfolobus solfataricus. J Bacteriol *183*, 3866-3874.

Cigan, A. M., Foiani, M., Hannig, E. M., and Hinnebusch, A. G. (1991). Complex formation by positive and negative translational regulators of GCN4. Mol Cell Biol *11*, 3217-3228.

Claude, A. (1943). Vol 10, Lancaster, The Jaques Cattell Press).

Coelho, P. S., Bryan, A. C., Kumar, A., Shadel, G. S., and Snyder, M. (2002). A novel mitochondrial protein, Tar1p, is encoded on the antisense strand of the nuclear 25S rDNA. Genes Dev *16*, 2755-2760.

Cote, C. A., Greer, C. L., and Peculis, B. A. (2002). Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast. Rna *8*, 786-797.

Cote, C. A., and Peculis, B. A. (2001). Role of the ITS2-proximal stem and evidence for indirect recognition of processing sites in pre-rRNA processing in yeast. Nucleic Acids Res *29*, 2106-2116.

Crick, F. H. (1958). On protein synthesis. Symp Soc Exp Biol, 138-163.

Crick, F. H., Barnett, L., Brenner, S., and Watts-Tobin, R. J. (1961). General nature of the genetic code for proteins. Nature *192*, 1227-1232.

Culver, G. M. (2003). Assembly of the 30S ribosomal subunit. Biopolymers *68*, 234-249.

Cunningham, P. R., Richard, R. B., Weitzmann, C. J., Nurse, K., and Ofengand, J. (1991). The absence of modified nucleotides affects both in vitro assembly and in vitro function of the 30S ribosomal subunit of Escherichia coli. Biochimie *73*, 789-796.

Dabbs, E. R. (1978). Mutational alterations in 50 proteins of the Escherichia coli ribosome. Mol Gen Genet *165*, 73-78.

Dabbs, E. R. (1991). Mutants lacking individual ribosomal proteins as a tool to investigate ribosomal properties. Biochimie *73*, 639-645.

Dahlberg, A. E., Dahlberg, J. E., Lund, E., Tokimatsu, H., Rabson, A. B., Calvert, P. C., Reynolds, F., and Zahalak, M. (1978). Processing of the 5' end of Escherichia coli 16S ribosomal RNA. Proc Natl Acad Sci U S A *75*, 3598-3602.

Davies, C., Bussiere, D. E., Golden, B. L., Porter, S. J., Ramakrishnan, V., and White, S. W. (1998). Ribosomal proteins S5 and L6: high-resolution crystal structures and roles in protein synthesis and antibiotic resistance. J Mol Biol *279*, 873-888.

Decatur, W. A., and Fournier, M. J. (2002). rRNA modifications and ribosome function. Trends Biochem Sci *27*, 344-351.

Decatur, W. A., and Fournier, M. J. (2003). RNA-guided nucleotide modification of ribosomal and other RNAs. J Biol Chem *278*, 695-698.

Deloche, O., de la Cruz, J., Kressler, D., Doere, M., and Linder, P. (2004). A membrane transport defect leads to a rapid attenuation of translation initiation in Saccharomyces cerevisiae. Mol Cell *13*, 357-366.

Demianova, M., Formosa, T. G., and Ellis, S. R. (1996). Yeast proteins related to the p40/laminin receptor precursor are essential components of the 40 S ribosomal subunit. J Biol Chem *271*, 11383-11391.

Dennis, P. P., and Omer, A. (2005). Small non-coding RNAs in Archaea. Curr Opin Microbiol *8*, 685-694.

Deshmukh, M., Tsay, Y. F., Paulovich, A. G., and Woolford, J. L., Jr. (1993). Yeast ribosomal protein L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S ribosomal subunits. Mol Cell Biol *13*, 2835-2845.

Dez, C., and Tollervey, D. (2004). Ribosome synthesis meets the cell cycle. Curr Opin Microbiol *7*, 631-637.

Dlakic, M., and Tollervey, D. (2004). The Noc proteins involved in ribosome synthesis and export contain divergent HEAT repeats. Rna *10*, 351-354.

Dragon, F., Gallagher, J. E., Compagnone-Post, P. A., Mitchell, B. M., Porwancher, K. A., Wehner, K. A., Wormsley, S., Settlage, R. E., Shabanowitz, J., Osheim, Y., et al. (2002). A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature *417*, 967-970.

Dresios, J., Panopoulos, P., Frantziou, C. P., and Synetos, D. (2001). Yeast ribosomal protein deletion mutants possess altered peptidyltransferase activity and different sensitivity to cycloheximide. Biochemistry *40*, 8101-8108.

Dunn, J. M., and Wong, K. P. (1979a). Molecular mechanism of in vitro 30 S ribosome assembly. I. Conformational changes of the 16 S RNA. J Biol Chem *254*, 7705-7711.

Dunn, J. M., and Wong, K. P. (1979b). Molecular mechanism of in vitro 30 S ribosome assembly. II. Conformational changes of ribosomal proteins. J Biol Chem *254*, 7712-7716.

Dunn, J. M., and Wong, K. P. (1979c). Structure and stability of the reconstituted intermediate particles involved in the in vitro self-assembly of the 30S ribosomal subunit. Biochemistry *18*, 4380-4385.

Durovic, P., and Dennis, P. P. (1994). Separate pathways for excision and processing of 16S and 23S rRNA from the primary rRNA operon transcript from the hyperthermophilic archaebacterium Sulfolobus acidocaldarius: similarities to eukaryotic rRNA processing. Mol Microbiol *13*, 229-242.

Dutca, L. M., Jagannathan, I., Grondek, J. F., and Culver, G. M. (2007). Temperature-dependent RNP conformational rearrangements: analysis of binary complexes of primary binding proteins with 16 S rRNA. J Mol Biol *368*, 853-869.

Errera, M., Hell, A., and Perry, R. P. (1961). The role of the nucleolus in ribonucleic acid- and protein synthesis. II. Amino acid incorporation into normal and nucleolar inactivated HeLa cells. Biochim Biophys Acta *49*, 58-63.

Eustice, D. C., and Wilhelm, J. M. (1984a). Fidelity of the eukaryotic codon-anticodon interaction: interference by aminoglycoside antibiotics. Biochemistry *23*, 1462-1467.

Eustice, D. C., and Wilhelm, J. M. (1984b). Mechanisms of action of aminoglycoside antibiotics in eucaryotic protein synthesis. Antimicrob Agents Chemother *26*, 53-60.

Faber, A. W., Van Dijk, M., Raue, H. A., and Vos, J. C. (2002). Ngl2p is a Ccr4p-like RNA nuclease essential for the final step in 3'-end processing of 5.8S rRNA in Saccharomyces cerevisiae. Rna *8*, 1095-1101.

Faber, A. W., Vos, H. R., Vos, J. C., and Raue, H. A. (2006). 5'-end formation of yeast 5.8SL rRNA is an endonucleolytic event. Biochem Biophys Res Commun *345*, 796-802.

Fath, S., Kobor, M. S., Philippi, A., Greenblatt, J., and Tschochner, H. (2004). Dephosphorylation of RNA polymerase I by Fcp1p is required for efficient rRNA synthesis. J Biol Chem *279*, 25251-25259.

Fatica, A., and Tollervey, D. (2002). Making ribosomes. Curr Opin Cell Biol *14*, 313-318.

Fewell, S. W., and Woolford, J. L., Jr. (1999). Ribosomal protein S14 of Saccharomyces cerevisiae regulates its expression by binding to RPS14B pre-mRNA and to 18S rRNA. Mol Cell Biol *19*, 826-834.

Fourmy, D., Recht, M. I., Blanchard, S. C., and Puglisi, J. D. (1996). Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science *274*, 1367-1371.

Fournier, M. J., and Maxwell, E. S. (1993). The nucleolar snRNAs: catching up with the spliceosomal snRNAs. Trends Biochem Sci *18*, 131-135.

Frey, S., Richter, R. P., and Gorlich, D. (2006). FG-rich repeats of nuclear pore proteins form a three-dimensional meshwork with hydrogel-like properties. Science *314*, 815-817.

Fromont-Racine, M., Senger, B., Saveanu, C., and Fasiolo, F. (2003). Ribosome assembly in eukaryotes. Gene *313*, 17-42.

Gabashvili, I. S., Agrawal, R. K., Spahn, C. M., Grassucci, R. A., Svergun, D. I., Frank, J., and Penczek, P. (2000). Solution structure of the E. coli 70S ribosome at 11.5 A resolution. Cell *100*, 537-549.

Gadal, O., Strauss, D., Kessl, J., Trumpower, B., Tollervey, D., and Hurt, E. (2001). Nuclear export of 60s ribosomal subunits depends on Xpo1p and requires a nuclear export sequence-containing factor, Nmd3p, that associates with the large subunit protein Rpl10p. Mol Cell Biol *21*, 3405-3415.

Gallagher, J. E., Dunbar, D. A., Granneman, S., Mitchell, B. M., Osheim, Y., Beyer, A. L., and Baserga, S. J. (2004). RNA polymerase I transcription and pre-rRNA processing are linked by specific SSU processome components. Genes Dev *18*, 2506-2517.

Ganot, P., Bortolin, M. L., and Kiss, T. (1997a). Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. Cell *89*, 799-809.

Ganot, P., Caizergues-Ferrer, M., and Kiss, T. (1997b). The family of box ACA small nucleolar RNAs is defined by an evolutionarily conserved secondary structure and ubiquitous sequence elements essential for RNA accumulation. Genes Dev *11*, 941-956.

Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141-147.

Gavrilova, L. P., Ivanov, D. A., and Spirin, A. S. (1966). Studies on the structure of ribosomes. 3. Stepwise unfolding of the 50 s particles without loss of ribosomal protein. J Mol Biol *16*, 473-489.

Geerlings, T. H., Faber, A. W., Bister, M. D., Vos, J. C., and Raue, H. A. (2003). Rio2p, an evolutionarily conserved, low abundant protein kinase essential for processing of 20 S Pre-rRNA in Saccharomyces cerevisiae. J Biol Chem *278*, 22537-22545.

Geerlings, T. H., Vos, J. C., and Raue, H. A. (2000). The final step in the formation of 25S rRNA in Saccharomyces cerevisiae is performed by 5'-->3' exonucleases. Rna *6*, 1698-1703.

Gegenheimer, P., and Apirion, D. (1980). Precursors to 16S and 23S ribosomal RNA from a ribonuclear III-strain of Escherichia coli contain intact RNase III processing sites. Nucleic Acids Res *8*, 1873-1891.

Gegenheimer, P., Watson, N., and Apirion, D. (1977). Multiple pathways for primary processing of ribosomal RNA in Escherichia coli. J Biol Chem *252*, 3064-3073.

Geiduschek, E. P., and Kassavetis, G. A. (2001). The RNA polymerase III transcription apparatus. J Mol Biol *310*, 1-26.

Gerbi, S. A., Borovjagin, A. V., and Lange, T. S. (2003). The nucleolus: a site of ribonucleoprotein maturation. Curr Opin Cell Biol *15*, 318-325.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature *418*, 387-391.

Giavalisco, P., Wilson, D., Kreitler, T., Lehrach, H., Klose, J., Gobom, J., and Fucini, P. (2005). High heterogeneity within the ribosomal proteins of the Arabidopsis thaliana 80S ribosome. Plant Mol Biol *57*, 577-591.

Gietz, R. D., and Sugino, A. (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene *74*, 527-534.

Ginsburg, D., and Steitz, J. A. (1975). The 30 S ribosomal precursor RNA from Escherichia coli. A primary transcript containing 23 S, 16 S, and 5 S sequences. J Biol Chem *250*, 5647-5654.

Gleizes, P. E., Noaillac-Depeyre, J., Leger-Silvestre, I., Teulieres, F., Dauxois, J. Y., Pommet, D., Azum-Gelade, M. C., and Gas, N. (2001). Ultrastructural localization of rRNA shows defective nuclear export of preribosomes in mutants of the Nup82p complex. J Cell Biol *155*, 923-936.

Glotz, C., and Brimacombe, R. (1980). An experimentally-derived model for the secondary structure of the 16S ribosomal RNA from Escherichia coli. Nucleic Acids Res *8*, 2377-2395.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., et al. (1996). Life with 6000 genes. Science *274*, 546, 563-547.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Grainger, R. M., and Maizels, N. (1980). Dictyostelium ribosomal RNA is processed during transcription. Cell *20*, 619-623.

Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., Schafer, T., Kuster, B., Tschochner, H., Tollervey, D., et al. (2002). 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. Mol Cell *10*, 105-115.

Granneman, S., and Baserga, S. J. (2004). Ribosome biogenesis: of knobs and RNA processing. Exp Cell Res *296*, 43-50.

Granneman, S., Bernstein, K. A., Bleichert, F., and Baserga, S. J. (2006). Comprehensive mutational analysis of yeast DEXD/H box RNA helicases required for small ribosomal subunit synthesis. Mol Cell Biol *26*, 1183-1194.

Granneman, S., Nandineni, M. R., and Baserga, S. J. (2005). The putative NTPase Fap7 mediates cytoplasmic 20S pre-rRNA processing through a direct interaction with Rps14. Mol Cell Biol *25*, 10352-10364.

Green, R., and Noller, H. F. (1999). Reconstitution of functional 50S ribosomes from in vitro transcripts of Bacillus stearothermophilus 23S rRNA. Biochemistry *38*, 1772-1779.

Gregory, S. T., and Dahlberg, A. E. (1999). Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. J Mol Biol *289*, 827-834.

Grummt, I. (2003). Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. Genes Dev *17*, 1691-1702.

Gu, S. Q., Peske, F., Wieden, H. J., Rodnina, M. V., and Wintermeyer, W. (2003). The signal recognition particle binds to protein L23 at the peptide exit of the Escherichia coli ribosome. Rna *9*, 566-573.

Gutell, R. R., Weiser, B., Woese, C. R., and Noller, H. F. (1985). Comparative anatomy of 16-S-like ribosomal RNA. Prog Nucleic Acid Res Mol Biol *32*, 155-216.

Hadjiolov, A. A. (1984-85). The Nucleolus and Ribosome Biogenesis, Springer-Verlag).

Hadjiolova, K. V., Nicoloso, M., Mazan, S., Hadjiolov, A. A., and Bachellerie, J. P. (1993). Alternative pre-rRNA processing pathways in human cells and their alteration by cycloheximide inhibition of protein synthesis. Eur J Biochem *212*, 211-215.

Haeusler, R. A., and Engelke, D. R. (2006). Spatial organization of transcription by RNA polymerase III. Nucleic Acids Res *34*, 4826-4836.

Hage, A. E., and Tollervey, D. (2004). A surfeit of factors: why is ribosome assembly so much more complicated in eukaryotes than bacteria? RNA Biol *1*, 10-15.

Haguenau, F. (1958). The ergastoplasm: Its history, ultrastructure and biochemistry. Int Rev Cytol, 425–483.

Halic, M., Becker, T., Frank, J., Spahn, C. M., and Beckmann, R. (2005). Localization and dynamic behavior of ribosomal protein L30e. Nat Struct Mol Biol *12*, 467-468.

Hall, D. B., Wade, J. T., and Struhl, K. (2006). An HMG protein, Hmo1, associates with promoters of many ribosomal protein genes and throughout the rRNA gene locus in Saccharomyces cerevisiae. Mol Cell Biol *26*, 3672-3679.

Hamilton, T. L., Stoneley, M., Spriggs, K. A., and Bushell, M. (2006). TOPs and their regulation. Biochem Soc Trans *34*, 12-16.

Hartwell, L. H., and McLaughlin, C. S. (1969). A mutant of yeast apparently defective in the initiation of protein synthesis. Proc Natl Acad Sci U S A *62*, 468-474.

Hayes, F., and Vasseur, M. (1976). Processing of the 17-S Escherichia coli precursor RNA in the 27-S pre-ribosomal particle. Eur J Biochem *61*, 433-442.

Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C. (1958a). Dependence of amino acid binding to soluble ribonucleic acid on cytidine triphosphate. Biochim Biophys Acta *29*, 460-461.

Hecht, L. I., Zamecnik, P. C., Stephenson, M. L., and Scott, J. F. (1958b). Nucleoside tri-phosphates as precursors of ribonucleic acid end groups in a mammalian system. J Biol Chem *233*, 954-963.

Hedges, J., West, M., and Johnson, A. W. (2005). Release of the export adapter, Nmd3p, from the 60S ribosomal subunit requires Rpl10p and the cytoplasmic GTPase Lsg1p. Embo J *24*, 567-579.

Held, W. A., Ballou, B., Mizushima, S., and Nomura, M. (1974). Assembly mapping of 30 S ribosomal proteins from Escherichia coli. Further studies. J Biol Chem *249*, 3103-3111.

Held, W. A., Mizushima, S., and Nomura, M. (1973). Reconstitution of Escherichia coli 30 S ribosomal subunits from purified molecular components. J Biol Chem *248*, 5720-5730.

Henras, A., Henry, Y., Bousquet-Antonelli, C., Noaillac-Depeyre, J., Gelugne, J. P., and Caizergues-Ferrer, M. (1998). Nhp2p and Nop10p are essential for the function of H/ACA snoRNPs. Embo J *17*, 7078-7090.

Henras, A. K., Bertrand, E., and Chanfreau, G. (2004). A cotranscriptional model for 3'-end processing of the Saccharomyces cerevisiae pre-ribosomal RNA precursor. Rna *10*, 1572-1585.

Henry, Y., Wood, H., Morrissey, J. P., Petfalski, E., Kearsey, S., and Tollervey, D. (1994). The 5' end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. Embo J *13*, 2452-2463.

Herold, M., and Nierhaus, K. H. (1987). Incorporation of six additional proteins to complete the assembly map of the 50 S subunit from Escherichia coli ribosomes. J Biol Chem *262*, 8826-8833.

Herold, M., Nowotny, V., Dabbs, E. R., and Nierhaus, K. H. (1986). Assembly analysis of ribosomes from a mutant lacking the assembly-initiator protein L24: lack of L24 induces temperature sensitivity. Mol Gen Genet *203*, 281-287.

Hinshaw, J. E., Carragher, B. O., and Milligan, R. A. (1992). Architecture and design of the nuclear pore complex. Cell *69*, 1133-1141.

Ho, J. H., and Johnson, A. W. (1999). NMD3 encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in Saccharomyces cerevisiae. Mol Cell Biol *19*, 2389-2399.

Ho, J. H., Kallstrom, G., and Johnson, A. W. (2000). Nmd3p is a Crm1p-dependent adapter protein for nuclear export of the large ribosomal subunit. J Cell Biol *151*, 1057-1066.

Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C. (1958). A soluble ribonucleic acid intermediate in protein synthesis. J Biol Chem *231*, 241-257.

Hoang, L., Fredrick, K., and Noller, H. F. (2004). Creating ribosomes with an all-RNA 30S subunit P site. Proc Natl Acad Sci U S A *101*, 12439-12443.

Hofmann, S., and Miller, O. L., Jr. (1977). Visualization of ribosomal ribonucleic acid synthesis in a ribonuclease III-Deficient strain of Escherichia coli. J Bacteriol *132*, 718-722.

Holmes, K. L., and Culver, G. M. (2004). Mapping structural differences between 30S ribosomal subunit assembly intermediates. Nat Struct Mol Biol *11*, 179-186.

Holmes, K. L., and Culver, G. M. (2005). Analysis of conformational changes in 16 S rRNA during the course of 30 S subunit assembly. J Mol Biol *354*, 340-357.

Hornstein, E., Tang, H., and Meyuhas, O. (2001). Mitogenic and nutritional signals are transduced into translational efficiency of TOP mRNAs. Cold Spring Harb Symp Quant Biol *66*, 477-484.

Hosokawa, K., Fujimura, R. K., and Nomura, M. (1966). Reconstitution of functionally active ribosomes from inactive subparticles and proteins. Proc Natl Acad Sci U S A *55*, 198-204.

Houseley, J., LaCava, J., and Tollervey, D. (2006). RNA-quality control by the exosome. Nat Rev Mol Cell Biol *7*, 529-539.

Hughes, J. M. (1996). Functional base-pairing interaction between highly conserved elements of U3 small nucleolar RNA and the small ribosomal subunit RNA. J Mol Biol *259*, 645-654.

Hughes, J. M., and Ares, M., Jr. (1991). Depletion of U3 small nucleolar RNA inhibits cleavage in the 5' external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. Embo J *10*, 4231-4239.

Hurt, E., Hannus, S., Schmelzl, B., Lau, D., Tollervey, D., and Simos, G. (1999). A novel in vivo assay reveals inhibition of ribosomal nuclear export in ran-cycle and nucleoporin mutants. J Cell Biol *144*, 389-401.

Huttenhofer, A. (2006). RNomics: identification and function of small non-protein-coding RNAs in model organisms. Cold Spring Harb Symp Quant Biol *71*, 135-140.

Huttenhofer, A., and Vogel, J. (2006). Experimental approaches to identify non-coding RNAs. Nucleic Acids Res *34*, 635-646.

Intine, R. V., Good, L., and Nazar, R. N. (1999). Essential structural features in the Schizosaccharomyces pombe pre-rRNA 5' external transcribed spacer. J Mol Biol *286*, 695-708.

Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J Bacteriol *153*, 163-168.

Jacob, F., and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol *3*, 318-356.

Jakovljevic, J., de Mayolo, P. A., Miles, T. D., Nguyen, T. M., Leger-Silvestre, I., Gas, N., and Woolford, J. L., Jr. (2004). The carboxy-terminal extension of yeast ribosomal protein S14 is necessary for maturation of 43S preribosomes. Mol Cell *14*, 331-342.

Jimenez, A., and Davies, J. (1980). Expression of a transposable antibiotic resistance element in Saccharomyces. Nature *287*, 869-871.

Johnson, A. W. (1997). Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. Mol Cell Biol *17*, 6122-6130.

Johnson, A. W., Lund, E., and Dahlberg, J. (2002). Nuclear export of ribosomal subunits. Trends Biochem Sci *27*, 580-585.

Joseph, N., Krauskopf, E., Vera, M. I., and Michot, B. (1999). Ribosomal internal transcribed spacer 2 (ITS2) exhibits a common core of secondary structure in vertebrates and yeast. Nucleic Acids Res *27*, 4533-4540.

Kallstrom, G., Hedges, J., and Johnson, A. (2003). The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. Mol Cell Biol *23*, 4344-4355.

Karbstein, K., Jonas, S., and Doudna, J. A. (2005). An essential GTPase promotes assembly of preribosomal RNA processing complexes. Mol Cell *20*, 633-643.

Kempers-Veenstra, A. E., Oliemans, J., Offenberg, H., Dekker, A. F., Piper, P. W., Planta, R. J., and Klootwijk, J. (1986). 3'-End formation of transcripts from the yeast rRNA operon. Embo J *5*, 2703-2710.

Khaitovich, P., Tenson, T., Kloss, P., and Mankin, A. S. (1999). Reconstitution of functionally active Thermus aquaticus large ribosomal subunits with in vitro-transcribed rRNA. Biochemistry *38*, 1780-1788.

Khanna-Gupta, A., and Ware, V. C. (1989). Nucleocytoplasmic transport of ribosomes in a eukaryotic system: is there a facilitated transport process? Proc Natl Acad Sci U S A *86*, 1791-1795.

King, T. C., Sirdeshmukh, R., and Schlessinger, D. (1984). RNase III cleavage is obligate for maturation but not for function of Escherichia coli pre-23S rRNA. Proc Natl Acad Sci U S A *81*, 185-188.

King, T. H., Liu, B., McCully, R. R., and Fournier, M. J. (2003). Ribosome structure and activity are altered in cells lacking snoRNPs that form pseudouridines in the peptidyl transferase center. Mol Cell *11*, 425-435.

Kiseleva, E., Goldberg, M. W., Allen, T. D., and Akey, C. W. (1998). Active nuclear pore complexes in Chironomus: visualization of transporter configurations related to mRNP export. J Cell Sci *111 (Pt 2)*, 223-236.

Kiss, T. (2004). Biogenesis of small nuclear RNPs. J Cell Sci *117*, 5949-5951.

Kiss-Laszlo, Z., Henry, Y., Bachellerie, J. P., Caizergues-Ferrer, M., and Kiss, T. (1996). Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell *85*, 1077-1088.

Kiss-Laszlo, Z., Henry, Y., and Kiss, T. (1998). Sequence and structural elements of methylation guide snoRNAs essential for site-specific ribose methylation of pre-rRNA. Embo J *17*, 797-807.

Kitakawa, M., and Isono, K. (1991). The mitochondrial ribosomes. Biochimie *73*, 813-825.

Kjems, J., and Garrett, R. A. (1988). Novel splicing mechanism for the ribosomal RNA intron in the archaebacterium Desulfurococcus mobilis. Cell *54*, 693-703.

Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M. (2000). rRNA operon copy number reflects ecological strategies of bacteria. Appl Environ Microbiol *66*, 1328-1333.

Klein, D. J., Moore, P. B., and Steitz, T. A. (2004). The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit. J Mol Biol *340*, 141-177.

Kobayashi, T., and Ganley, A. R. (2005). Recombination regulation by transcription-induced cohesin dissociation in rDNA repeats. Science *309*, 1581-1584.

Kobayashi, T., Heck, D. J., Nomura, M., and Horiuchi, T. (1998). Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. Genes Dev *12*, 3821-3830.

Kobayashi, T., Nomura, M., and Horiuchi, T. (2001). Identification of DNA cis elements essential for expansion of ribosomal DNA repeats in Saccharomyces cerevisiae. Mol Cell Biol *21*, 136-147.

Kohler, A., and Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. Nat Rev Mol Cell Biol *8*, 761-773.

Kramer, G., Rauch, T., Rist, W., Vorderwulbecke, S., Patzelt, H., Schulze-Specking, A., Ban, N., Deuerling, E., and Bukau, B. (2002). L23 protein functions as a chaperone docking site on the ribosome. Nature *419*, 171-174.

Kressler, D., Linder, P., and de La Cruz, J. (1999). Protein trans-acting factors involved in ribosome biogenesis in Saccharomyces cerevisiae. Mol Cell Biol *19*, 7897-7912.

Kruger, T., Zentgraf, H., and Scheer, U. (2007). Intranucleolar sites of ribosome biogenesis defined by the localization of early binding ribosomal proteins. J Cell Biol *177*, 573-578.

Kruiswijk, T., de Hey, J. T., and Planta, R. J. (1978a). Modification of yeast ribosomal proteins. Phosphorylation. Biochem J *175*, 213-219.

Kruiswijk, T., Kunst, A., Planta, R. J., and Mager, W. H. (1978b). Modification of yeast ribosomal proteins. Methylation. Biochem J *175*, 221-225.

Kruiswijk, T., and Planta, R. J. (1974). Analysis of the protein composition of yeast ribosomal subunits by two-dimensional polyacrylamide gel electrophoresis. Mol Biol Rep *1*, 409-415.

Kruiswijk, T., Planta, R. J., and Mager, W. H. (1978c). Quantitative analysis of the protein composition of yeast ribosomes. Eur J Biochem *83*, 245-252.

Kuai, L., Fang, F., Butler, J. S., and Sherman, F. (2004). Polyadenylation of rRNA in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *101*, 8581-8586.

Kufel, J., Allmang, C., Petfalski, E., Beggs, J., and Tollervey, D. (2003). Lsm Proteins are required for normal processing and stability of ribosomal RNAs. J Biol Chem *278*, 2147-2156.

Kufel, J., Dichtl, B., and Tollervey, D. (1999). Yeast Rnt1p is required for cleavage of the pre-ribosomal RNA in the 3' ETS but not the 5' ETS. Rna *5*, 909-917.

LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E., Jacquier, A., and Tollervey, D. (2005). RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. Cell *121*, 713-724.

Lafontaine, D., Delcour, J., Glasser, A. L., Desgres, J., and Vandenhaute, J. (1994). The DIM1 gene responsible for the conserved m6(2)Am6(2)A dimethylation in the 3'-terminal loop of 18 S rRNA is essential in yeast. J Mol Biol *241*, 492-497.

Lafontaine, D., Vandenhaute, J., and Tollervey, D. (1995). The 18S rRNA dimethylase Dim1p is required for pre-ribosomal RNA processing in yeast. Genes Dev *9*, 2470-2481.

Lafontaine, D. L., Preiss, T., and Tollervey, D. (1998). Yeast 18S rRNA dimethylase Dim1p: a quality control mechanism in ribosome synthesis? Mol Cell Biol *18*, 2360-2370.

Lafontaine, D. L., and Tollervey, D. (1998). Birth of the snoRNPs: the evolution of the modification-guide snoRNAs. Trends Biochem Sci *23*, 383-388.

Lafontaine, D. L., and Tollervey, D. (2001). The function and synthesis of ribosomes. Nat Rev Mol Cell Biol *2*, 514-520.

Lake, J. A. (1985). Evolving ribosome structure: domains in archaebacteria, eubacteria, eocytes and eukaryotes. Annu Rev Biochem *54*, 507-530.

Lamborg, M. R., and Zamecnik, P. C. (1960). Amino acid incorporation into protein by extracts of E. coli. Biochim Biophys Acta *42*, 206-211.

Lamontagne, B., and Elela, S. A. (2004). Evaluation of the RNA determinants for bacterial and yeast RNase III binding and cleavage. J Biol Chem *279*, 2231-2241.

Lamontagne, B., Ghazal, G., Lebars, I., Yoshizawa, S., Fourmy, D., and Elela, S. A. (2003). Sequence dependence of substrate recognition and cleavage by yeast RNase III. J Mol Biol *327*, 985-1000.

Lamontagne, B., Larose, S., Boulanger, J., and Elela, S. A. (2001). The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. Curr Issues Mol Biol *3*, 71-78.

Lane, B. G. (1998). Historical Perspectives on RNA Nucleoside Modifications. In Modification and Editing of RNA, H. G. a. R. Benne, ed. (ASM Press).

LaRonde-LeBlanc, N., and Wlodawer, A. (2005). A family portrait of the RIO kinases. J Biol Chem *280*, 37297-37300.

Lascaris, R. F., Groot, E., Hoen, P. B., Mager, W. H., and Planta, R. J. (2000). Different roles for abf1p and a T-rich promoter element in nucleosome organization of the yeast RPS28A gene. Nucleic Acids Res *28*, 1390-1396.

Lascaris, R. F., Mager, W. H., and Planta, R. J. (1999). DNA-binding requirements of the yeast protein Rap1p as selected in silico from ribosomal protein gene promoter sequences. Bioinformatics *15*, 267-277.

Lavergne, J. P., Marzouki, A., Reboud, J. P., and Reboud, A. M. (1988). Reconstitution of the active rat liver 60 S ribosomal subunit from different preparations of core particles and split proteins. FEBS Lett *236*, 345-351.

Lebreton, A., Saveanu, C., Decourty, L., Rain, J. C., Jacquier, A., and Fromont-Racine, M. (2006). A functional network involved in the recycling of nucleocytoplasmic pre-60S factors. J Cell Biol *173*, 349-360.

Lecompte, O., Ripp, R., Thierry, J. C., Moras, D., and Poch, O. (2002). Comparative analysis of ribosomal proteins in complete genomes: an example of reductive evolution at the domain scale. Nucleic Acids Res *30*, 5382-5390.

Lee, K., Bernstein, J. A., and Cohen, S. N. (2002). RNase G complementation of rne null mutation identifies functional interrelationships with RNase E in Escherichia coli. Mol Microbiol *43*, 1445-1456.

Lee, Y., and Nazar, R. N. (1997). Ribosomal 5 S rRNA maturation in Saccharomyces cerevisiae. J Biol Chem *272*, 15206-15212.

Lee, Y., and Nazar, R. N. (2003). Terminal structure mediates 5 S rRNA stability and integration during ribosome biogenesis. J Biol Chem *278*, 6635-6641.

Leger-Silvestre, I., Caffrey, J. M., Dawaliby, R., Alvarez-Arias, D. A., Gas, N., Bertolone, S. J., Gleizes, P. E., and Ellis, S. R. (2005). Specific Role for Yeast Homologs of the Diamond Blackfan Anemia-associated Rps19 Protein in Ribosome Synthesis. J Biol Chem *280*, 38177-38185.

Leger-Silvestre, I., Milkereit, P., Ferreira-Cerca, S., Saveanu, C., Rousselle, J. C., Choesmel, V., Guinefoleau, C., Gas, N., and Gleizes, P. E. (2004). The ribosomal protein Rps15p is required for nuclear exit of the 40S subunit precursors in yeast. Embo J *23*, 2336-2347.

Leger-Silvestre, I., Trumtel, S., Noaillac-Depeyre, J., and Gas, N. (1999). Functional compartmentalization of the nucleus in the budding yeast Saccharomyces cerevisiae. Chromosoma *108*, 103-113.

Lengyel, P., Speyer, J. F., and Ochoa, S. (1961). Synthetic polynucleotides and the amino acid code. Proc Natl Acad Sci U S A *47*, 1936-1942.

Lerman, M. I., Spirin, A. S., Gavrilova, L. P., and Golov, V. F. (1966). Studies on the structure of ribosomes. II. Stepwise dissociation of protein from ribosomes by caesium chloride and the re-assembly of ribosome-like particles. J Mol Biol *15*, 268-281.

Lesnyak, D. V., Osipiuk, J., Skarina, T., Sergiev, P. V., Bogdanov, A. A., Edwards, A., Savchenko, A., Joachimiak, A., and Dontsova, O. A. (2007). Methyltransferase that modifies guanine 966 of the 16 S rRNA: functional identification and tertiary structure. J Biol Chem *282*, 5880-5887.

Lesnyak, D. V., Sergiev, P. V., Bogdanov, A. A., and Dontsova, O. A. (2006). Identification of Escherichia coli m2G methyltransferases: I. the ycbY gene encodes a methyltransferase specific for G2445 of the 23 S rRNA. J Mol Biol *364*, 20-25.

Leulliot, N., Quevillon-Cheruel, S., Graille, M., van Tilbeurgh, H., Leeper, T. C., Godin, K. S., Edwards, T. E., Sigurdsson, S. T., Rozenkrants, N., Nagel, R. J., et al. (2004). A new alpha-helical extension promotes RNA binding by the dsRBD of Rnt1p RNAse III. Embo J *23*, 2468-2477.

Li, B., Vilardell, J., and Warner, J. R. (1996). An RNA structure involved in feedback regulation of splicing and of translation is critical for biological fitness. Proc Natl Acad Sci U S A *93*, 1596-1600.

Li, H. D., Zagorski, J., and Fournier, M. J. (1990). Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in Saccharomyces cerevisiae. Mol Cell Biol *10*, 1145-1152.

Li, Z., and Deutscher, M. P. (1995). The tRNA processing enzyme RNase T is essential for maturation of 5S RNA. Proc Natl Acad Sci U S A *92*, 6883-6886.

Li, Z., Pandit, S., and Deutscher, M. P. (1999a). Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. Rna *5*, 139-146.

Li, Z., Pandit, S., and Deutscher, M. P. (1999b). RNase G (CafA protein) and RNase E are both required for the 5' maturation of 16S ribosomal RNA. Embo J *18*, 2878-2885.

Liang, W. Q., and Fournier, M. J. (1995). U14 base-pairs with 18S rRNA: a novel snoRNA interaction required for rRNA processing. Genes Dev *9*, 2433-2443.

Liang, W. Q., and Fournier, M. J. (1997). Synthesis of functional eukaryotic ribosomal RNAs in trans: development of a novel in vivo rDNA system for dissecting ribosome biogenesis. Proc Natl Acad Sci U S A *94*, 2864-2868.

Liljas, A. (1991). Comparative biochemistry and biophysics of ribosomal proteins. Int Rev Cytol *124*, 103-136.

Lindahl, L. (1975). Intermediates and time kinetics of the in vivo assembly of Escherichia coli ribosomes. J Mol Biol *92*, 15-37.

Lindahl, L., Archer, R. H., and Zengel, J. M. (1992). A new rRNA processing mutant of Saccharomyces cerevisiae. Nucleic Acids Res *20*, 295-301.

Lindstrom, M. S., Deisenroth, C., and Zhang, Y. (2007). Putting a finger on growth surveillance: insight into MDM2 zinc finger-ribosomal protein interactions. Cell Cycle *6*, 434-437.

Lischwe, M. A., Ochs, R. L., Reddy, R., Cook, R. G., Yeoman, L. C., Tan, E. M., Reichlin, M., and Busch, H. (1985). Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in NG,NG-dimethylarginine. J Biol Chem *260*, 14304-14310.

Littlefield, J. W., and Dunn, D. B. (1958a). Natural occurrence of thymine and three methylated adenine bases in several ribonucleic acids. Nature *181*, 254-255.

Littlefield, J. W., and Dunn, D. B. (1958b). The occurrence and distribution of thymine and three methylated-adenine bases in ribonucleic acids from several sources. Biochem J *70*, 642-651.

Loar, J. W., Seiser, R. M., Sundberg, A. E., Sagerson, H. J., Ilias, N., Zobel-Thropp, P., Craig, E. A., and Lycan, D. E. (2004). Genetic and biochemical interactions among Yar1, Ltv1 and Rps3 define novel links between environmental stress and ribosome biogenesis in Saccharomyces cerevisiae. Genetics *168*, 1877-1889.

Londei, P., Teixido, J., Acca, M., Cammarano, P., and Amils, R. (1986). Total reconstitution of active large ribosomal subunits of the thermoacidophilic archaebacterium Sulfolobus solfataricus. Nucleic Acids Res *14*, 2269-2285.

Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953-961.

Lovgren, J. M., and Wikstrom, P. M. (2001). The rlmB gene is essential for formation of Gm2251 in 23S rRNA but not for ribosome maturation in Escherichia coli. J Bacteriol *183*, 6957-6960.

Lucioli, A., Presutti, C., Ciafre, S., Caffarelli, E., Fragapane, P., and Bozzoni, I. (1988). Gene dosage alteration of L2 ribosomal protein genes in Saccharomyces cerevisiae: effects on ribosome synthesis. Mol Cell Biol *8*, 4792-4798.

Lygerou, Z., Allmang, C., Tollervey, D., and Seraphin, B. (1996). Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP in vitro. Science *272*, 268-270.

Lykke-Andersen, J., Aagaard, C., Semionenkov, M., and Garrett, R. A. (1997). Archaeal introns: splicing, intercellular mobility and evolution. Trends Biochem Sci *22*, 326-331.

MacRae, I. J., and Doudna, J. A. (2007). Ribonuclease revisited: structural insights into ribonuclease III family enzymes. Curr Opin Struct Biol *17*, 138-145.

Mager, W. H., Planta, R. J., Ballesta, J. G., Lee, J. C., Mizuta, K., Suzuki, K., Warner, J. R., and Woolford, J. (1997). A new nomenclature for the cytoplasmic ribosomal proteins of Saccharomyces cerevisiae. Nucleic Acids Res *25*, 4872-4875.

Maki, J. A., and Culver, G. M. (2005). Recent developments in factor-facilitated ribosome assembly. Methods *36*, 313-320.

Maki, J. A., Schnobrich, D. J., and Culver, G. M. (2002). The DnaK chaperone system facilitates 30S ribosomal subunit assembly. Mol Cell *10*, 129-138.

Maki, J. A., Southworth, D. R., and Culver, G. M. (2003). Demonstration of the role of the DnaK chaperone system in assembly of 30S ribosomal subunits using a purified in vitro system. Rna *9*, 1418-1421.

Mandiyan, V., Tumminia, S., Wall, J. S., Hainfeld, J. F., and Boublik, M. (1989). Protein-induced conformational changes in 16 S ribosomal RNA during the initial assembly steps of the Escherichia coli 30 S ribosomal subunit. J Mol Biol *210*, 323-336.

Mandiyan, V., Tumminia, S. J., Wall, J. S., Hainfeld, J. F., and Boublik, M. (1991). Assembly of the Escherichia coli 30S ribosomal subunit reveals protein-dependent folding of the 16S rRNA domains. Proc Natl Acad Sci U S A *88*, 8174-8178.

Mangiarotti, G., and Chiaberge, S. (1997). Reconstitution of functional eukaryotic ribosomes from Dictyostelium discoideum ribosomal proteins and RNA. J Biol Chem *272*, 19682-19687.

Martin, D. E., Soulard, A., and Hall, M. N. (2004). TOR regulates ribosomal protein gene expression via PKA and the Forkhead transcription factor FHL1. Cell *119*, 969-979.

Marygold, S. J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G., Harrison, P., Yu, Z., Kenmochi, N., Kaufman, T. C., et al. (2007). The ribosomal protein genes and Minute loci of Drosophila melanogaster. Genome Biol *8*, R216.

Mauro, V. P., and Edelman, G. M. (2002). The ribosome filter hypothesis. Proc Natl Acad Sci U S A *99*, 12031-12036.

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R. K., DiCorleto, P. E., and Fox, P. L. (2003). Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. Cell *115*, 187-198.

McCloskey, J. A., and Rozenski, J. (2005). The Small Subunit rRNA Modification Database. Nucleic Acids Res *33*, D135-138.

Michot, B., Joseph, N., Mazan, S., and Bachellerie, J. P. (1999). Evolutionarily conserved structural features in the ITS2 of mammalian pre-rRNAs and potential interactions with the snoRNA U8 detected by comparative analysis of new mouse sequences. Nucleic Acids Res *27*, 2271-2282.

Milkereit, P., Gadal, O., Podtelejnikov, A., Trumtel, S., Gas, N., Petfalski, E., Tollervey, D., Mann, M., Hurt, E., and Tschochner, H. (2001). Maturation and intranuclear transport of pre-ribosomes requires Noc proteins. Cell *105*, 499-509.

Milkereit, P., Kuhn, H., Gas, N., and Tschochner, H. (2003a). The pre-ribosomal network. Nucleic Acids Res *31*, 799-804.

Milkereit, P., Strauss, D., Bassler, J., Gadal, O., Kuhn, H., Schutz, S., Gas, N., Lechner, J., Hurt, E., and Tschochner, H. (2003b). A Noc complex specifically involved in the formation and nuclear export of ribosomal 40 S subunits. J Biol Chem *278*, 4072-4081.

Miller, O. L., Jr., and Beatty, B. R. (1969). Visualization of nucleolar genes. Science *164*, 955-957.

Miller, O. L., Jr., Hamkalo, B. A., and Thomas, C. A., Jr. (1970). Visualization of bacterial genes in action. Science *169*, 392-395.

Misra, T. K., and Apirion, D. (1979). RNase E, an RNA processing enzyme from Escherichia coli. J Biol Chem *254*, 11154-11159.

Mitchell, J. R., Cheng, J., and Collins, K. (1999). A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. Mol Cell Biol *19*, 567-576.

Mitchell, P., Petfalski, E., Houalla, R., Podtelejnikov, A., Mann, M., and Tollervey, D. (2003). Rrp47p is an exosome-associated protein required for the 3' processing of stable RNAs. Mol Cell Biol *23*, 6982-6992.

Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M., and Tollervey, D. (1997). The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-->5' exoribonucleases. Cell *91*, 457-466.

Mitchell, P., Petfalski, E., and Tollervey, D. (1996). The 3' end of yeast 5.8S rRNA is generated by an exonuclease processing mechanism. Genes Dev *10*, 502-513.

Mizushima, S., and Nomura, M. (1970). Assembly mapping of 30S ribosomal proteins from E. coli. Nature *226*, 1214.

Moll, I., Grill, S., Gualerzi, C. O., and Blasi, U. (2002). Leaderless mRNAs in bacteria: surprises in ribosomal recruitment and translational control. Mol Microbiol *43*, 239-246.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-663.

Moritz, M., Paulovich, A. G., Tsay, Y. F., and Woolford, J. L., Jr. (1990). Depletion of yeast ribosomal proteins L16 or rp59 disrupts ribosome assembly. J Cell Biol *111*, 2261-2274.

Moritz, M., Pulaski, B. A., and Woolford, J. L., Jr. (1991). Assembly of 60S ribosomal subunits is perturbed in temperature-sensitive yeast mutants defective in ribosomal protein L16. Mol Cell Biol *11*, 5681-5692.

Morrissey, J. P., and Tollervey, D. (1993). Yeast snR30 is a small nucleolar RNA required for 18S rRNA synthesis. Mol Cell Biol *13*, 2469-2477.

Morrissey, J. P., and Tollervey, D. (1995). Birth of the snoRNPs: the evolution of RNase MRP and the eukaryotic pre-rRNA-processing system. Trends Biochem Sci *20*, 78-82.

Moss, T. (2004). At the crossroads of growth control; making ribosomal RNA. Curr Opin Genet Dev *14*, 210-217.

Moss, T., Langlois, F., Gagnon-Kugler, T., and Stefanovsky, V. (2007). A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. Cell Mol Life Sci *64*, 29-49.

Mougey, E. B., O'Reilly, M., Osheim, Y., Miller, O. L., Jr., Beyer, A., and Sollner-Webb, B. (1993). The terminal balls characteristic of eukaryotic rRNA transcription units in chromatin spreads are rRNA processing complexes. Genes Dev *7*, 1609-1619.

Moy, T. I., and Silver, P. A. (1999). Nuclear export of the small ribosomal subunit requires the ran-GTPase cycle and certain nucleoporins. Genes Dev *13*, 2118-2133.

Munro, H. N., Baliga, B. S., and Pronczuk, A. W. (1968). In vitro inhibition of peptide synthesis and GTP hydrolysis by cycloheximide and reversal of inhibition by glutathione. Nature *219*, 944-946.

Muto, A., Ehresmann, C., Fellner, P., and Zimmermann, R. A. (1974). RNA-protein interactions in the ribosome. I. Characterization and ribonuclease digestion of 16 S RNA-ribosomal protein complexes. J Mol Biol *86*, 411-432.

Nagel, R., and Ares, M., Jr. (2000). Substrate recognition by a eukaryotic RNase III: the double-stranded RNA-binding domain of Rnt1p selectively binds RNA containing a 5'-AGNN-3' tetraloop. Rna *6*, 1142-1156.

Nakao, A., Yoshihama, M., and Kenmochi, N. (2004). RPG: the Ribosomal Protein Gene database. Nucleic Acids Res *32*, D168-170.

Nariai, M., Tanaka, T., Okada, T., Shirai, C., Horigome, C., and Mizuta, K. (2005). Synergistic defect in 60S ribosomal subunit assembly caused by a mutation of Rrs1p, a ribosomal protein L11-binding protein, and 3'-extension of 5S rRNA in Saccharomyces cerevisiae. Nucleic Acids Res *33*, 4553-4562.

Neumann, E., and Rosenheck, K. (1972). Permeability changes induced by electric impulses in vesicular membranes. J Membr Biol *10*, 279-290.

Neville, M., and Rosbash, M. (1999). The NES-Crm1p export pathway is not a major mRNA export route in Saccharomyces cerevisiae. Embo J *18*, 3746-3756.

Ni, J., Tien, A. L., and Fournier, M. J. (1997). Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. Cell *89*, 565-573.

Nicholson, A. W. (1999). Function, mechanism and regulation of bacterial ribonucleases. FEMS Microbiol Rev *23*, 371-390.

Nierhaus, K. H. (1980a). Analysis of the assembly and function of the 50S subunit from Escherichia coli ribosomes by reconstitution. In Ribosomes, G. Chambliss, ed. (Baltimore, MD, University Park Press), pp. 267-297.

Nierhaus, K. H. (1980b). The assembly of the prokaryotic ribosome. Biosystems *12*, 273-282.

Nierhaus, K. H. (1991). The assembly of prokaryotic ribosomes. Biochimie *73*, 739-755.

Nierhaus, K. H., Bordasch, K., and Homann, H. E. (1973). Ribosomal proteins. 43. In vivo assembly of Escherichia coli ribosomal proteins. J Mol Biol *74*, 587-597.

Nierhaus, K. H., and Dohme, F. (1974). Total reconstitution of functionally active 50S ribosomal subunits from Escherichia coli. Proc Natl Acad Sci U S A *71*, 4713-4717.

Nierhaus, K. H. W., Daniel N (2004). Protein synthesis and ribosome structure : translating the genome. Weinheim : Wiley-VCH.

Nirenberg, M. W., and Matthaei, J. H. (1961). The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. Proc Natl Acad Sci U S A *47*, 1588-1602.

Nishiyama, T., Yamamoto, H., Uchiumi, T., and Nakashima, N. (2007). Eukaryotic ribosomal protein RPS25 interacts with the conserved loop region in a dicistroviral intergenic internal ribosome entry site. Nucleic Acids Res *35*, 1514-1521.

Nissan, T. A., Bassler, J., Petfalski, E., Tollervey, D., and Hurt, E. (2002). 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. Embo J *21*, 5539-5547.

Nissan, T. A., Galani, K., Maco, B., Tollervey, D., Aebi, U., and Hurt, E. (2004). A pre-ribosome with a tadpole-like structure functions in ATP-dependent maturation of 60S subunits. Mol Cell *15*, 295-301.

Nitta, I., Kamada, Y., Noda, H., Ueda, T., and Watanabe, K. (1998). Reconstitution of peptide bond formation with Escherichia coli 23S ribosomal RNA domains. Science *281*, 666-669.

Noller, H. F., Hoffarth, V., and Zimniak, L. (1992). Unusual resistance of peptidyl transferase to protein extraction procedures. Science *256*, 1416-1419.

Noller, H. F., Yusupov, M. M., Yusupova, G. Z., Baucom, A., Lieberman, K., Lancaster, L., Dallas, A., Fredrick, K., Earnest, T. N., and Cate, J. H. (2001). Structure of the ribosome at 5.5 A resolution and its interactions with functional ligands. Cold Spring Harb Symp Quant Biol *66*, 57-66.

Nomura, M. (1970). Bacterial Ribosome. Bacteriol Rev *34*, 228-277.

Nomura, M. (1999). Regulation of ribosome biosynthesis in Escherichia coli and Saccharomyces cerevisiae: diversity and common principles. J Bacteriol *181*, 6857-6864.

Nomura, M. (2001). Ribosomal RNA genes, RNA polymerases, nucleolar structures, and synthesis of rRNA in the yeast Saccharomyces cerevisiae. Cold Spring Harb Symp Quant Biol *66*, 555-565.

Nomura, M., and Erdmann, V. A. (1970). Reconstitution of 50S ribosomal subunits from dissociated molecular components. Nature *228*, 744-748.

Nomura, M., and Traub, P. (1968). Structure and function of Escherichia coli ribosomes. 3. Stoichiometry and rate of the reconstitution of ribosomes from subribosomal particles and split proteins. J Mol Biol *34*, 609-619.

Nomura, M., Traub, P., and Bechmann, H. (1968). Hybrid 30S ribosomal particles reconstituted from components of different bacterial origins. Nature *219*, 793-799.

O'Farrell, H. C., Pulicherla, N., Desai, P. M., and Rife, J. P. (2006). Recognition of a complex substrate by the KsgA/Dim1 family of enzymes has been conserved throughout evolution. Rna *12*, 725-733.

Obrig, T. G., Culp, W. J., McKeehan, W. L., and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J Biol Chem *246*, 174-181.

Ochs, R. L., Lischwe, M. A., Spohn, W. H., and Busch, H. (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell *54*, 123-133.

Oeffinger, M., Dlakic, M., and Tollervey, D. (2004). A pre-ribosome-associated HEAT-repeat protein is required for export of both ribosomal subunits. Genes Dev *18*, 196-209.

Osheim, Y. N., French, S. L., Keck, K. M., Champion, E. A., Spasov, K., Dragon, F., Baserga, S. J., and Beyer, A. L. (2004). Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to being cleaved from nascent transcripts in Saccharomyces cerevisiae. Mol Cell *16*, 943-954.

Peculis, B. A. (1997). The sequence of the 5' end of the U8 small nucleolar RNA is critical for 5.8S and 28S rRNA maturation. Mol Cell Biol *17*, 3702-3713.

Peculis, B. A., and Greer, C. L. (1998). The structure of the ITS2-proximal stem is required for pre-rRNA processing in yeast. Rna *4*, 1610-1622.

Pederson, T. (1998). The plurifunctional nucleolus. Nucleic Acids Res *26*, 3871-3876.

Pemberton, L. F., and Paschal, B. M. (2005). Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic *6*, 187-198.

Perry, R. P. (1960). On the nucleolar and nuclear dependence of cytoplasmic RNA synthesis in HeLa cells. Exp Cell Res *20*, 216-220.

Perry, R. P., and Errera, M. (1961). The role of the nucleolus in ribonucleic acid-and protein synthesis. I. Incorporation of cytidine into normal and nucleolar inactivated HeLa cells. Biochim Biophys Acta *49*, 47-57.

Perry, R. P., and Meyuhas, O. (1990). Translational control of ribosomal protein production in mammalian cells. Enzyme *44*, 83-92.

Petfalski, E., Dandekar, T., Henry, Y., and Tollervey, D. (1998). Processing of the precursors to small nucleolar RNAs and rRNAs requires common components. Mol Cell Biol *18*, 1181-1189.

Piekna-Przybylska, D., Decatur, W. A., and Fournier, M. J. (2007). New bioinformatic tools for analysis of nucleotide modifications in eukaryotic rRNA. Rna *13*, 305-312.

Piper, P. W., Bellatin, J. A., and Lockheart, A. (1983). Altered maturation of sequences at the 3' terminus of 5S gene transcripts in a Saccharomyces cerevisiae mutant that lacks a RNA processing endonuclease. Embo J *2*, 353-359.

Planta, R. J. (1997). Regulation of ribosome synthesis in yeast. Yeast *13*, 1505-1518.

Powers, T., Changchien, L. M., Craven, G. R., and Noller, H. F. (1988). Probing the assembly of the 3' major domain of 16 S ribosomal RNA. Quaternary interactions involving ribosomal proteins S7, S9 and S19. J Mol Biol *200*, 309-319.

Powers, T., Daubresse, G., and Noller, H. F. (1993). Dynamics of in vitro assembly of 16 S rRNA into 30 S ribosomal subunits. J Mol Biol *232*, 362-374.

Powers, T., and Noller, H. F. (1995a). Hydroxyl radical footprinting of ribosomal proteins on 16S rRNA. Rna *1*, 194-209.

Powers, T., and Noller, H. F. (1995b). A temperature-dependent conformational rearrangement in the ribosomal protein S4.16 S rRNA complex. J Biol Chem *270*, 1238-1242.

Radu, A., Moore, M. S., and Blobel, G. (1995). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215-222.

Ramagopal, S. (1992). Are eukaryotic ribosomes heterogeneous? Affirmations on the horizon. Biochem Cell Biol *70*, 269-272.

Ramagopal, S., and Ennis, H. L. (1981). Regulation of synthesis of cell-specific ribosomal proteins during differentiation of Dictyostelium discoideum. Proc Natl Acad Sci U S A *78*, 3083-3087.

Raska, I., Koberna, K., Malinsky, J., Fidlerova, H., and Masata, M. (2004). The nucleolus and transcription of ribosomal genes. Biol Cell *96*, 579-594.

Reboud, A. M., Buisson, M., Amoros, M. J., and Reboud, J. P. (1972). Partial in vitro reconstitution of active 40S ribosomal subunits from rat liver. Biochem Biophys Res Commun *46*, 2012-2018.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol *17*, 1030-1032.

Roberts, R. B. (1958). Microsomal Particles and Protein Synthesis. (New York, Pergamon Press, Inc).

Rodriguez, M. S., Dargemont, C., and Stutz, F. (2004). Nuclear export of RNA. Biol Cell *96*, 639-655.

Rohl, R., and Nierhaus, K. H. (1982). Assembly map of the large subunit (50S) of Escherichia coli ribosomes. Proc Natl Acad Sci U S A *79*, 729-733.

Rotenberg, M. O., Moritz, M., and Woolford, J. L., Jr. (1988). Depletion of Saccharomyces cerevisiae ribosomal protein L16 causes a decrease in 60S ribosomal subunits and formation of half-mer polyribosomes. Genes Dev *2*, 160-172.

Rouquette, J., Choesmel, V., and Gleizes, P. E. (2005). Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells. Embo J *24*, 2862-2872.

Rout, M. P., Blobel, G., and Aitchison, J. D. (1997). A distinct nuclear import pathway used by ribosomal proteins. Cell *89*, 715-725.

Roy, M. K., Singh, B., Ray, B. K., and Apirion, D. (1983). Maturation of 5-S rRNA: ribonuclease E cleavages and their dependence on precursor sequences. Eur J Biochem *131*, 119-127.

Rozenski, J., Crain, P. F., and McCloskey, J. A. (1999). The RNA Modification Database: 1999 update. Nucleic Acids Res *27*, 196-197.

Rudra, D., Zhao, Y., and Warner, J. R. (2005). Central role of Ifh1p-Fhl1p interaction in the synthesis of yeast ribosomal proteins. Embo J *24*, 533-542.

Russell, A. G., Ebhardt, H., and Dennis, P. P. (1999). Substrate requirements for a novel archaeal endonuclease that cleaves within the 5' external transcribed spacer of Sulfolobus acidocaldarius precursor rRNA. Genetics *152*, 1373-1385.

Saeboe-Larssen, S., Lyamouri, M., Merriam, J., Oksvold, M. P., and Lambertsson, A. (1998). Ribosomal protein insufficiency and the minute syndrome in Drosophila: a dose-response relationship. Genetics *148*, 1215-1224.

Saltzman, L., and Apirion, D. (1976). Binding of erythromycin to the 50S ribosomal subunit is affected by alterations in the 30S ribosomal subunit. Mol Gen Genet *143*, 301-306.

Samaha, R. R., O'Brien, B., O'Brien, T. W., and Noller, H. F. (1994). Independent in vitro assembly of a ribonucleoprotein particle containing the 3' domain of 16S rRNA. Proc Natl Acad Sci U S A *91*, 7884-7888.

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press).

Sanchez, M. E., Londei, P., and Amils, R. (1996). Total reconstitution of active small ribosomal subunits of the extreme halophilic archaeon Haloferax mediterranei. Biochim Biophys Acta *1292*, 140-144.

Sanchez, M. E., Urena, D., Amils, R., and Londei, P. (1990). In vitro reassembly of active large ribosomal subunits of the halophilic archaebacterium Haloferax mediterranei. Biochemistry *29*, 9256-9261.

Saveanu, C., Bienvenu, D., Namane, A., Gleizes, P. E., Gas, N., Jacquier, A., and Fromont-Racine, M. (2001). Nog2p, a putative GTPase associated with pre-60S subunits and required for late 60S maturation steps. Embo J *20*, 6475-6484.

Schafer, T., Maco, B., Petfalski, E., Tollervey, D., Bottcher, B., Aebi, U., and Hurt, E. (2006). Hrr25-dependent phosphorylation state regulates organization of the pre-40S subunit. Nature *441*, 651-655.

Schafer, T., Strauss, D., Petfalski, E., Tollervey, D., and Hurt, E. (2003). The path from nucleolar 90S to cytoplasmic 40S pre-ribosomes. Embo J *22*, 1370-1380.

Schawalder, S. B., Kabani, M., Howald, I., Choudhury, U., Werner, M., and Shore, D. (2004). Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Ifh1. Nature *432*, 1058-1061.

Scheer, U., and Benavente, R. (1990). Functional and dynamic aspects of the mammalian nucleolus. Bioessays *12*, 14-21.

Scheer, U., and Hock, R. (1999). Structure and function of the nucleolus. Curr Opin Cell Biol *11*, 385-390.

Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Gorlich, D., Ponstingl, H., and Bischoff, F. R. (1997). Yrb4p, a yeast ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. Embo J *16*, 6237-6249.

Schlessinger, D. (1979). Processing of ribosomal RNA transcripts in bacteria. In Ribosomes, J. e. a. Davies, eds., ed. (University Park Press, MD), pp. 767-781.

Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res *18*, 3091-3092.

Schmitt, M. E., and Clayton, D. A. (1994). Characterization of a unique protein component of yeast RNase MRP: an RNA-binding protein with a zinc-cluster domain. Genes Dev *8*, 2617-2628.

Schneider, D. A., Michel, A., Sikes, M. L., Vu, L., Dodd, J. A., Salgia, S., Osheim, Y. N., Beyer, A. L., and Nomura, M. (2007). Transcription elongation by RNA polymerase I is linked to efficient rRNA processing and ribosome assembly. Mol Cell *26*, 217-229.

Seiser, R. M., Sundberg, A. E., Wollam, B. J., Zobel-Thropp, P., Baldwin, K., Spector, M. D., and Lycan, D. E. (2006). Ltv1 is required for efficient nuclear export of the ribosomal small subunit in Saccharomyces cerevisiae. Genetics *174*, 679-691.

Senger, B., Lafontaine, D. L., Graindorge, J. S., Gadal, O., Camasses, A., Sanni, A., Garnier, J. M., Breitenbach, M., Hurt, E., and Fasiolo, F. (2001). The nucle(ol)ar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis. Mol Cell *8*, 1363-1373.

Sergiev, P. V., Lesnyak, D. V., Bogdanov, A. A., and Dontsova, O. A. (2006). Identification of Escherichia coli m2G methyltransferases: II. The ygjO gene encodes a methyltransferase specific for G1835 of the 23 S rRNA. J Mol Biol *364*, 26-31.

Sharma, K., Venema, J., and Tollervey, D. (1999). The 5' end of the 18S rRNA can be positioned from within the mature rRNA. Rna *5*, 678-686.

Shine, J., and Dalgarno, L. (1974). The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc Natl Acad Sci U S A *71*, 1342-1346.

Shuai, K., and Warner, J. R. (1991). A temperature sensitive mutant of Saccharomyces cerevisiae defective in pre-rRNA processing. Nucleic Acids Res *19*, 5059-5064.

Siekevitz, P. (1952). Uptake of radioactive alanine in vitro into the proteins of rat liver fractions. J Biol Chem *195*, 549-565.

Siekevitz, P., and Zamecnick, P. C. (1951). In vitro incorporation of 1-14C-DL-alanine into proteins of rat liver granular fractions. Fed Proc, 246.

Sirdeshmukh, R., Krych, M., and Schlessinger, D. (1985). Escherichia coli 23S ribosomal RNA truncated at its 5' terminus. Nucleic Acids Res *13*, 1185-1192.

Sirdeshmukh, R., and Schlessinger, D. (1985). Ordered processing of Escherichia coli 23S rRNA in vitro. Nucleic Acids Res *13*, 5041-5054.

Smith, J. D., and Dunn, D. B. (1959). An additional sugar component of ribonucleic acids. Biochim Biophys Acta *31*, 573-575.

Smith, J. S., and Boeke, J. D. (1997). An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev *11*, 241-254.

Smits, P., Smeitink, J. A., van den Heuvel, L. P., Huynen, M. A., and Ettema, T. J. (2007). Reconstructing the evolution of the mitochondrial ribosomal proteome. Nucleic Acids Res *35*, 4686-4703.

Spahn, C. M., Beckmann, R., Eswar, N., Penczek, P. A., Sali, A., Blobel, G., and Frank, J. (2001). Structure of the 80S ribosome from Saccharomyces cerevisiae--tRNA-ribosome and subunit-subunit interactions. Cell *107*, 373-386.

Spahn, C. M., Gomez-Lorenzo, M. G., Grassucci, R. A., Jorgensen, R., Andersen, G. R., Beckmann, R., Penczek, P. A., Ballesta, J. P., and Frank, J. (2004). Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation. Embo J *23*, 1008-1019.

Spirin, A. S. (2000). Ribosomes, Springer).

Spirin, A. S., and Belitsina, N. V. (1966). Biological activity of the re-assembled ribosome-like particles. J Mol Biol *15*, 282-283.

Spirin, A. S., Lerman, M. I., Gavrilova, L. P., and Belitsina, N. V. (1966). [Reconstruction of biologically active ribosomes from protein-poor ribonucleoprotein particles and ribosomal protein]. Biokhimiia *31*, 424-430.

Srivastava, A. K., and Schlessinger, D. (1988). Coregulation of processing and translation: mature 5' termini of Escherichia coli 23S ribosomal RNA form in polysomes. Proc Natl Acad Sci U S A *85*, 7144-7148.

Srivastava, A. K., and Schlessinger, D. (1990). Mechanism and regulation of bacterial ribosomal RNA processing. Annu Rev Microbiol *44*, 105-129.

Staehelin, T., and Meselson, M. (1966). In vitro recovery o ribosomes and of synthetic activity from synthetically inactive ribosomal subunits. J Mol Biol *16*, 245-249.

Stage-Zimmermann, T., Schmidt, U., and Silver, P. A. (2000). Factors affecting nuclear export of the 60S ribosomal subunit in vivo. Mol Biol Cell *11*, 3777-3789.

Stanners, C. P. (1966). The effect of cycloheximide on polyribosomes from hamster cells. Biochem Biophys Res Commun *22*, 758-764.

Stansfield, I., Jones, K. M., Herbert, P., Lewendon, A., Shaw, W. V., and Tuite, M. F. (1998). Missense translation errors in Saccharomyces cerevisiae. J Mol Biol *282*, 13-24.

Stern, S., Powers, T., Changchien, L. M., and Noller, H. F. (1989). RNA-protein interactions in 30S ribosomal subunits: folding and function of 16S rRNA. Science *244*, 783-790.

Stevens, A., Hsu, C. L., Isham, K. R., and Larimer, F. W. (1991). Fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in Saccharomyces cerevisiae lacking 5'----3' exoribonuclease 1. J Bacteriol *173*, 7024-7028.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A. G., Aguilera, A., Struhl, K., Reed, R., and Hurt, E. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature *417*, 304-308.

Strawn, L. A., Shen, T., Shulga, N., Goldfarb, D. S., and Wente, S. R. (2004). Minimal nuclear pore complexes define FG repeat domains essential for transport. Nat Cell Biol *6*, 197-206.

Svergun, D. I., and Nierhaus, K. H. (2000). A map of protein-rRNA distribution in the 70 S Escherichia coli ribosome. J Biol Chem *275*, 14432-14439.

Synetos, D., Dabeva, M. D., and Warner, J. R. (1992). The yeast ribosomal protein S7 and its genes. J Biol Chem *267*, 3008-3013.

Synetos, D., Frantziou, C. P., and Alksne, L. E. (1996). Mutations in yeast ribosomal proteins S28 and S4 affect the accuracy of translation and alter the sensitivity of the ribosomes to paromomycin. Biochim Biophys Acta *1309*, 156-166.

Tabb, A. L., Utsugi, T., Wooten-Kee, C. R., Sasaki, T., Edling, S. A., Gump, W., Kikuchi, Y., and Ellis, S. R. (2001). Genes encoding ribosomal proteins Rps0A/B of Saccharomyces cerevisiae interact with TOM1 mutants defective in ribosome synthesis. Genetics *157*, 1107-1116.

Tabb-Massey, A., Caffrey, J. M., Logsden, P., Taylor, S., Trent, J. O., and Ellis, S. R. (2003). Ribosomal proteins Rps0 and Rps21 of Saccharomyces cerevisiae have overlapping functions in the maturation of the 3' end of 18S rRNA. Nucleic Acids Res *31*, 6798-6805.

Talkington, M. W., Siuzdak, G., and Williamson, J. R. (2005). An assembly landscape for the 30S ribosomal subunit. Nature *438*, 628-632.

Tang, T. H., Rozhdestvensky, T. S., d'Orval, B. C., Bortolin, M. L., Huber, H., Charpentier, B., Branlant, C., Bachellerie, J. P., Brosius, J., and Huttenhofer, A. (2002). RNomics in Archaea reveals a further link between splicing of archaeal introns and rRNA processing. Nucleic Acids Res *30*, 921-930.

Thiry, M., and Lafontaine, D. L. (2005). Birth of a nucleolus: the evolution of nucleolar compartments. Trends Cell Biol *15*, 194-199.

Thomas, F., and Kutay, U. (2003). Biogenesis and nuclear export of ribosomal subunits in higher eukaryotes depend on the CRM1 export pathway. J Cell Sci *116*, 2409-2419.

Thompson, M., Haeusler, R. A., Good, P. D., and Engelke, D. R. (2003). Nucleolar clustering of dispersed tRNA genes. Science *302*, 1399-1401.

Tischendorf, G. W., Zeichhardt, H., and Stoffler, G. (1974a). Determination of the location of proteins L14, L17, L18, L19, L22, L23 on the surface of the 5oS ribosomal subunit of Escherichia coli by immune electron microscopy. Mol Gen Genet *134*, 187-208.

Tischendorf, G. W., Zeichhardt, H., and Stoffler, G. (1974b). Location of proteins S5, S13 and S14 on the surface of the 3oS ribosomal subunit from Escherichia coli as determined by immune electron microscopy. Mol Gen Genet *134*, 209-223.

Tischendorf, G. W., Zeichhardt, H., and Stoffler, G. (1975). Architecture of the Escherichia coli ribosome as determined by immune electron microscopy. Proc Natl Acad Sci U S A *72*, 4820-4824.

Tissieres, A., and Watson, J. D. (1958). Ribonucleoprotein particles from Escherichia coli. Nature *182*, 778-780.

Tissieres, A., Watson, J. D., Schlessinger, D., and Hollingworth, B. R. (1959). Ribonucleoprotein particles from Escherichia coli. J Mol Biol *1*, 221-233.

Tollervey, D. (1987). A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. Embo J *6*, 4169-4175.

Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., and Hurt, E. C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. Cell *72*, 443-457.

Trapman, J., and Planta, R. J. (1975). Detailed analysis of the ribosomal RNA synthesis in yeast. Biochim Biophys Acta *414*, 115-125.

Trapman, J., and Planta, R. J. (1976). Maturation of ribosomes in yeast. I Kinetic analysis by labelling of high molecular weight rRNA species. Biochim Biophys Acta *442*, 265-274.

Trapman, J., Planta, R. J., and Raue, H. A. (1976). Maturation of ribosomes in yeast. II. Position of the low molecular weight rRNA species in the maturation process. Biochim Biophys Acta *442*, 275-284.

Traub, P., Hosokawa, K., Craven, G. R., and Nomura, M. (1967). Structure and function of E. coli ribosomes, IV. Isolation and characterization of functionally active ribosomal proteins. Proc Natl Acad Sci U S A *58*, 2430-2436.

Traub, P., and Nomura, M. (1968a). Structure and function of E. coli ribosomes. V. Reconstitution of functionally active 30S ribosomal particles from RNA and proteins. Proc Natl Acad Sci U S A *59*, 777-784.

Traub, P., and Nomura, M. (1968b). Structure and function of Escherichia coli ribosomes. I. Partial fractionation of the functionally active ribosomal proteins and reconstitution of artificial subribosomal particles. J Mol Biol *34*, 575-593.

Traub, P., and Nomura, M. (1969). Structure and function of Escherichia coli ribosomes. VI. Mechanism of assembly of 30 s ribosomes studied in vitro. J Mol Biol *40*, 391-413.

Traub, P., Soll, D., and Nomura, M. (1968). Structure and function of Escherichia coli ribosomes. II. Translational fidelity and efficiency in protein synthesis of a protein-deficient subribosomal particle. J Mol Biol *34*, 595-608.

Trotta, C. R., Lund, E., Kahan, L., Johnson, A. W., and Dahlberg, J. E. (2003). Coordinated nuclear export of 60S ribosomal subunits and NMD3 in vertebrates. Embo J *22*, 2841-2851.

Tsay, Y. F., Shankweiler, G., Lake, J., and Woolford, J. L., Jr. (1994). Localization of Saccharomyces cerevisiae ribosomal protein L16 on the surface of 60 S ribosomal subunits by immunoelectron microscopy. J Biol Chem *269*, 7579-7586.

Udem, S. A., and Warner, J. R. (1973). The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. J Biol Chem *248*, 1412-1416.

Valasek, L., Mathew, A. A., Shin, B. S., Nielsen, K. H., Szamecz, B., and Hinnebusch, A. G. (2003). The yeast eIF3 subunits TIF32/a, NIP1/c, and eIF5 make critical connections with the 40S ribosome in vivo. Genes Dev *17*, 786-799.

Valentin, G. (1836). Repertorium für Anatomie und Physiologie,, Vol 1, Verlag von Veit und Comp. Berlin).

van Beekvelt, C. A., de Graaff-Vincent, M., Faber, A. W., van't Riet, J., Venema, J., and Raue, H. A. (2001a). All three functional domains of the large ribosomal subunit protein L25 are required for both early and late pre-rRNA processing steps in Saccharomyces cerevisiae. Nucleic Acids Res *29*, 5001-5008.

van Beekvelt, C. A., Jeeninga, R. E., van't Riet, J., Venema, J., and Raue, H. A. (2001b). Identification of cis-acting elements involved in 3'-end formation of Saccharomyces cerevisiae 18S rRNA. Rna *7*, 896-903.

van der Sande, C. A., Kulkens, T., Kramer, A. B., de Wijs, I. J., van Heerikhuizen, H., Klootwijk, J., and Planta, R. J. (1989). Termination of transcription by yeast RNA polymerase I. Nucleic Acids Res *17*, 9127-9146.

van der Sande, C. A., Kwa, M., van Nues, R. W., van Heerikhuizen, H., Raue, H. A., and Planta, R. J. (1992). Functional analysis of internal transcribed spacer 2 of Saccharomyces cerevisiae ribosomal DNA. J Mol Biol *223*, 899-910.

van Hoof, A., Lennertz, P., and Parker, R. (2000). Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast. Embo J *19*, 1357-1365.

van Nues, R. W., Rientjes, J. M., van der Sande, C. A., Zerp, S. F., Sluiter, C., Venema, J., Planta, R. J., and Raue, H. A. (1994). Separate structural elements within internal transcribed spacer 1 of Saccharomyces cerevisiae precursor ribosomal RNA direct the formation of 17S and 26S rRNA. Nucleic Acids Res *22*, 912-919.

van Nues, R. W., Venema, J., Rientjes, J. M., Dirks-Mulder, A., and Raue, H. A. (1995). Processing of eukaryotic pre-rRNA: the role of the transcribed spacers. Biochem Cell Biol *73*, 789-801.

Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., and Lafontaine, D. L. (2004). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. Rna *10*, 645-656.

Vanrobays, E., Gelugne, J. P., Gleizes, P. E., and Caizergues-Ferrer, M. (2003). Late cytoplasmic maturation of the small ribosomal subunit requires RIO proteins in Saccharomyces cerevisiae. Mol Cell Biol *23*, 2083-2095.

Vanrobays, E., Gleizes, P. E., Bousquet-Antonelli, C., Noaillac-Depeyre, J., Caizergues-Ferrer, M., and Gelugne, J. P. (2001). Processing of 20S pre-rRNA to 18S ribosomal RNA in yeast requires Rrp10p, an essential non-ribosomal cytoplasmic protein. Embo J *20*, 4204-4213.

Veldman, G. M., Klootwijk, J., de Regt, V. C., Planta, R. J., Branlant, C., Krol, A., and Ebel, J. P. (1981). The primary and secondary structure of yeast 26S rRNA. Nucleic Acids Res *9*, 6935-6952.

Venema, J., Henry, Y., and Tollervey, D. (1995). Two distinct recognition signals define the site of endonucleolytic cleavage at the 5'-end of yeast 18S rRNA. Embo J *14*, 4883-4892.

Venema, J., and Tollervey, D. (1995). Processing of pre-ribosomal RNA in Saccharomyces cerevisiae. Yeast *11*, 1629-1650.

Venema, J., and Tollervey, D. (1999). Ribosome synthesis in Saccharomyces cerevisiae. Annu Rev Genet *33*, 261-311.

Vilardell, J., and Warner, J. R. (1994). Regulation of splicing at an intermediate step in the formation of the spliceosome. Genes Dev *8*, 211-220.

Vilardell, J., and Warner, J. R. (1997). Ribosomal protein L32 of Saccharomyces cerevisiae influences both the splicing of its own transcript and the processing of rRNA. Mol Cell Biol *17*, 1959-1965.

Vilardell, J., Yu, S. J., and Warner, J. R. (2000). Multiple functions of an evolutionarily conserved RNA binding domain. Mol Cell *5*, 761-766.

Vioque, A., Pintor-Toro, J. A., and Palacian, E. (1982). Partial reconstitution of active eukaryotic ribosomes following dissociation with dimethylmaleic anhydride. J Biol Chem *257*, 6477-6480.

Vos, H. R., Faber, A. W., de Gier, M. D., Vos, J. C., and Raue, H. A. (2004). Deletion of the three distal S1 motifs of Saccharomyces cerevisiae Rrp5p abolishes pre-rRNA processing at site A(2) without reducing the production of functional 40S subunits. Eukaryot Cell *3*, 1504-1512.

Voth, W. P., Jiang, Y. W., and Stillman, D. J. (2003). New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids. Yeast *20*, 985-993.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793-1808.

Wade, J. T., Hall, D. B., and Struhl, K. (2004). The transcription factor Ifh1 is a key regulator of yeast ribosomal protein genes. Nature *432*, 1054-1058.

Wagner, R. (1835). Einige Bemerkungen und Fragen über das Keimbläschen (vesicula germinativa). Müller's Arch Anat Physiol Wissenschaftliche Med, 373-377.

Walker, S. C., and Engelke, D. R. (2006). Ribonuclease P: the evolution of an ancient RNA enzyme. Crit Rev Biochem Mol Biol *41*, 77-102.

Wang, R., and Brattain, M. G. (2007). The maximal size of protein to diffuse through the nuclear pore is larger than 60kDa. FEBS Lett *581*, 3164-3170.

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. Trends Biochem Sci *24*, 437-440.

Warner, J. R., Kumar, A., Udem, S. A., and Wu, R. S. (1972). Ribosomal proteins and the assembly of ribosomes in eukaryotes. Biochem J *129*, 29P-30P.

Weinstein, L. B., and Steitz, J. A. (1999). Guided tours: from precursor snoRNA to functional snoRNP. Curr Opin Cell Biol *11*, 378-384.

Weitzmann, C., Tumminia, S. J., Boublik, M., and Ofengand, J. (1991). A paradigm for local conformational control of function in the ribosome: binding of ribosomal protein S19 to Escherichia coli 16S rRNA in the presence of S7 is required for methylation of m2G966 and blocks methylation of m5C967 by their respective methyltransferases. Nucleic Acids Res *19*, 7089-7095.

West, M., Hedges, J. B., Chen, A., and Johnson, A. W. (2005). Defining the order in which Nmd3p and Rpl10p load onto nascent 60S ribosomal subunits. Mol Cell Biol *25*, 3802-3813.

Williamson, J. R. (2003). After the ribosome structures: how are the subunits assembled? Rna *9*, 165-167.

Wilson, D. N., Blaha, G., Connell, S. R., Ivanov, P. V., Jenke, H., Stelzl, U., Teraoka, Y., and Nierhaus, K. H. (2002). Protein synthesis at atomic resolution: mechanistics of translation in the light of highly resolved structures for the ribosome. Curr Protein Pept Sci *3*, 1-53.

Wilson, D. N., and Nierhaus, K. H. (2003). The ribosome through the looking glass. Angew Chem Int Ed Engl *42*, 3464-3486.

Wilson, D. N., and Nierhaus, K. H. (2005). Ribosomal proteins in the spotlight. Crit Rev Biochem Mol Biol *40*, 243-267.

Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Jr., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000). Structure of the 30S ribosomal subunit. Nature *407*, 327-339.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., et al. (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science *285*, 901-906.

Woese, C. R., Magrum, L. J., Gupta, R., Siegel, R. B., Stahl, D. A., Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J. J., and Noller, H. F. (1980). Secondary structure model for bacterial 16S ribosomal RNA: phylogenetic, enzymatic and chemical evidence. Nucleic Acids Res *8*, 2275-2293.

Wool, I. G. (1996). Extraribosomal functions of ribosomal proteins. Trends Biochem Sci *21*, 164-165.

Wrzesinski, J., Nurse, K., Bakin, A., Lane, B. G., and Ofengand, J. (1995). A dual-specificity pseudouridine synthase: an Escherichia coli synthase purified and cloned on the basis of its specificity for psi 746 in 23S RNA is also specific for psi 32 in tRNA(phe). Rna *1*, 437-448.

Wu, H., Henras, A., Chanfreau, G., and Feigon, J. (2004). Structural basis for recognition of the AGNN tetraloop RNA fold by the double-stranded RNA-binding domain of Rnt1p RNase III. Proc Natl Acad Sci U S A *101*, 8307-8312.

Yao, W., Roser, D., Kohler, A., Bradatsch, B., Bassler, J., and Hurt, E. (2007). Nuclear export of ribosomal 60S subunits by the general mRNA export receptor Mex67-Mtr2. Mol Cell *26*, 51-62.

Yeh, L. C., and Lee, J. C. (1990). Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from Saccharomyces cerevisiae. J Mol Biol *211*, 699-712.

Yeh, L. C., and Lee, J. C. (1992). Structure analysis of the 5' external transcribed spacer of the precursor ribosomal RNA from Saccharomyces cerevisiae. J Mol Biol *228*, 827-839.

Yeh, L. C., Thweatt, R., and Lee, J. C. (1990). Internal transcribed spacer 1 of the yeast precursor ribosomal RNA. Higher order structure and common structural motifs. Biochemistry *29*, 5911-5918.

Yeh, Y. C., Traut, R. R., and Lee, J. C. (1986). Protein topography of the 40 S ribosomal subunit from Saccharomyces cerevisiae as shown by chemical cross-linking. J Biol Chem *261*, 14148-14153.

Young, R. A., and Steitz, J. A. (1978). Complementary sequences 1700 nucleotides apart form a ribonuclease III cleavage site in Escherichia coli ribosomal precursor RNA. Proc Natl Acad Sci U S A *75*, 3593-3597.

Yusupov, M. M., Yusupova, G. Z., Baucom, A., Lieberman, K., Earnest, T. N., Cate, J. H., and Noller, H. F. (2001). Crystal structure of the ribosome at 5.5 A resolution. Science *292*, 883-896.

Zebarjadian, Y., King, T., Fournier, M. J., Clarke, L., and Carbon, J. (1999). Point mutations in yeast CBF5 can abolish in vivo pseudouridylation of rRNA. Mol Cell Biol *19*, 7461-7472.

Zeitler, B., and Weis, K. (2004). The FG-repeat asymmetry of the nuclear pore complex is dispensable for bulk nucleocytoplasmic transport in vivo. J Cell Biol *167*, 583-590.

Zhang, B., and Cech, T. R. (1997). Peptide bond formation by in vitro selected ribozymes. Nature *390*, 96-100.

Zhang, B., and Cech, T. R. (1998). Peptidyl-transferase ribozymes: trans reactions, structural characterization and ribosomal RNA-like features. Chem Biol *5*, 539-553.

Zhao, Y., McIntosh, K. B., Rudra, D., Schawalder, S., Shore, D., and Warner, J. R. (2006). Fine-structure analysis of ribosomal protein gene transcription. Mol Cell Biol *26*, 4853-4862.

Zimmermann, R. A. (2003). The double life of ribosomal proteins. Cell *115*, 130-132.

Zimmermann, R. A., Muto, A., and Mackie, G. A. (1974). RNA-protein interactions in the ribosome. II. Binding of ribosomal proteins to isolated fragments of the 16 S RNA. J Mol Biol *86*, 433-450.

PUBLICATIONS

Publications directly ensue from this work:

Ferreira-Cerca S, Pöll G, Gleizes PE, Tschochner H, Milkereit P. «**Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function**». Mol. Cell. 2005 Oct 28;20(2):263-75.

Ferreira-Cerca S, Pöll G, Kühn H, Neueder A, Jacob S, Tschochner H, Milkereit P. «**Analysis of the *in vivo* assembly pathway of eukaryotic 40S ribosomal proteins**». Mol. Cell. 2007 Nov 9;28(3):446-457.

ACKNOWLEDGEMENTS

First of all, I would like to thank the jury members for accepting to evaluate this work.

I owe my gratitude to my mentor Dr. Philipp Milkereit for his outstanding supervision, for his enthusiasm, comments, suggestions, and criticisms, for all his help to make this work successful. For his help to settle in Germany.

I would like to thank Prof. Dr. Pierre-Emmanuel Gleizes and Prof. Dr. Herbert Tschochner, my Ph.D. advisors, for their support, comments and suggestions.

I´m in debt to Prof. Dr. Herbert Tschochner for allowing me to work in his institute, for his contributions and help during the publication process, and for giving me the opportunity to participate to international meetings.

Gisela Pöll for her irreplaceable excellent technical assistance, and for all the generous help provided outside of the lab and the excellent food she offered me (greetings to your family).

Dr. Jean-Paul Gelugne, Prof. Dr. Pierre-Emmanuel Gleizes, Dr. Isabelle Léger-Silvestre, and Dr. Emmanuel Vanrobays for offering me the chance to work with them prior to my Ph.D. work, and for giving me an excellent and helpful scientific education.

My colleagues for sharing buffers and equipments. I would like to thank especially Jochen Gerber, Dr. Holger Kühn, and Anja Philippi for their constant support, scientific and non-scientific discussions and memorable moments inside and outside the lab.

Dr. Joachim Griesenbeck for correcting this manuscript, and for his constant enthusiasm and interest on this work.

Prof. Dr. Pierre-Emmanuel Gleizes, Dr. Isabelle Léger-Silvestre for sharing strains and results prior to publication.

Hannah Götze, Maité Hanot, Steffen Jakob, Stephan Jellbauer, Dr. Holger Kühn and Andreas Neueder for providing tools described or undescribed in this Ph.D. thesis.

Kristin Hergert, Anja Jacob, Gisela Pöll and Ulrike Stöckl for providing common media, buffers, tools for molecular biology and for their lab management.

Edith Aichinger and Dr. Helfried Mallow for all their help.

My friends for being always here despite the physical distance.

I´m deeply in debt to my familly for all their constant help, support and love they have offered me so far.

Special thanks to Anja Philippi for her love and for all the wonderfull moments we share …and for those still to come…

三人行 , 必有我师焉 : 择其善者而从之 , 其不善者而改之 。 - Confucius - The Analects.