Tandem affinity purification combined with inducible shRNA expression as a tool to study the maturation of macromolecular assemblies

A dissertation submitted to
ETH Zurich

for the degree of
Doctor of Sciences

presented by
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2010
Acknowledgments

An undertaking such as a thesis cannot be achieved alone. I am therefore very grateful to the following people.

I would like to thank Prof. Ulrike Kutay for accepting me in her lab, for advice and support during the thesis, and for promoting the fascinating research in human ribosome biogenesis.

Prof. Matthias Peter and Dr. Matthias Gstaiger acted as co-examiners for my thesis, and Prof. Arlen Johnson, Prof. Nenad Ban and Dr. Vikram Panse gave very valuable advice in the thesis committees meetings.

Two former lab members, Drs. Roland Koller-Eichhorn and Ivo Zemp, established many of the methods used in this dissertation and introduced me to them. Without their founding work, this thesis would not have been possible. In addition, I would like to thank especially Ivo for the very pleasant and fruitful collaboration in the Rrp12 publication.

Barbara Widmann, Thomas Wild, Eva Laurell and Yagmur Turgay accompanied me during my time in the Kutay lab. I greatly appreciate their friendship and support in everyday lab life! Barbara, Thomas and Yagmur helped me in correcting this thesis.

I would like to thank all members of the Kutay lab for making it a pleasant place to work, especially Caroline Ashiono for the technical assistance and Franziska Wandrey and Lukas Badertscher for continuing the studies in ribosome biogenesis.

Julia Steringer, Peter Blattmann, Laleh Bahrami, Michaela Gerber and Daniel Alper all contributed as students or apprentices to this thesis – and I hope that they have learned something as well during the time here.

Within the IBC, I relied heavily on the support of Peter Horvath, Joachim Hehl and Gabor Csucs (LMC) as well as of Anton Lehmann, Sonja Muggler, Katrin Peter, Catherine Brasseur, Daniela Tschümperlin, Marianne Chiesi, Eva Lekkas, Verena Haller, Monika Lienhard, Maria Cerdeira and the D-BIOL shop. Big thanks also to the IT pals Nico Graf and Roland Stuber!
Many thanks also to Alexander Wepf, Jens Pfannstiel, Sébastien Fribourg and Christian Spahn, our collaborators for mass spectrometry and structural biology.

Living with a biochemist is not always easy – I thank Carol Ribi, family and friends for being with me.
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Summary

Ribosomes are situated at a key point in every living organism, as they translate the information stored in the genome into proteins, the functional entities in cells. Ribosome biogenesis, the construction of ribosomes from RNA and ribosomal proteins, is thus fundamental. Knowledge of this process in human cells is however still sparse. So far, research on unicellular model organisms such as *E. coli* or *S. cerevisiae* has been instrumental in deciphering the mechanisms underlying ribosome biogenesis. To what extent the data obtained there can be extrapolated to human ribosome biogenesis is still unclear.

We set out to gather both descriptive and mechanistic data about the assembly of human ribosomes. At first, we adapted a tandem affinity purification method using *trans*-acting factors as bait proteins for efficient isolation of pre-ribosomal particles from human cell lines. By testing several different bait proteins, precursors of both ribosomal subunits could be isolated and their composition determined. Several of the identified proteins have homologues in yeast that were shown to be involved in ribosome biogenesis. In addition, new factors could be discovered. One of them, c21orf70, was further characterized and a role in human pre-40S maturation confirmed.

To understand the role of certain proteins in ribosome biogenesis, combining tandem affinity purification with simultaneous depletion of specific factors may of great help. We constructed such a system using inducible shRNA and used it to further clarify the role of the kinase Rio2, which was already shown to be involved in the biogenesis of the small subunit. Thus, we demonstrated that reduced levels of Rio2 led to an enrichment of Rrp12 on nascent 40S subunits. Rrp12 is a factor putatively required for nuclear export of pre-ribosomal particles. The need for Rio2 to release Rrp12 in the cytoplasm was confirmed by immunofluorescence. Tandem affinity purification was also the basis for the finding that the kinase CK2 is involved in 60S biogenesis. When using the GTPase GNL1 as bait, we found that it associates with and is phosphorylated by CK2 at Ser51. Mutating this residue to alanine increased the affinity of GNL1 to pre-60S particles, suggesting that the phosphorylation leads to dissociation of GNL1 from pre-ribosomal particles in the cytoplasm. We confirmed that by showing that GNL1, besides other 60S *trans*-acting factors, was enriched at the 60S peak on sucrose gradient centrifugation after depletion of the structural CK2 subunit, CK2β.

At last, we found a trimer containing the 60S *trans*-acting factors Tif6, the ribosomal protein Rpl23 and the β isoform of BCCIP (BRCA2 and Cdc1 interacting protein). BCCIP has been implicated in DNA double strand break repair and regulation of cell proliferation in human
cells, whereas the yeast homologue Bcp1p is necessary for nuclear export of the lipid kinase Mss4. We could show that overexpression of BCCIPβ, but not the other isoform BCCIPα, led to a 60S export defect in human cells. This indicates an isoform-specific role in 60S biogenesis.

In summary, the tandem affinity purification in combination with inducible protein depletion was proven to be a valuable tool for the human ribosome biogenesis pathway. Besides the identification of trans-acting factors, it also served as a starting point for investigations into molecular details of ribosome assembly.
Zusammenfassung


Auch die Hypothese dass die Proteinkinase CK2 and der Biogenese der 60S-Untereinheit beteiligt ist, basierte auf der hier beschriebenen TAP-Methode. Die GTPase GNL1 bildete einen Komplex mit CK2, und wird von dieser an Serin 51 phosphoryliert. Nachdem dieses
Serin zu Alanin mutiert wurde, um eine Phosphorylierung zu verunmöglichen, band GNL1 an prä-60S-Partikel. Dies ist ein Hinweis darauf, dass die Phosphorylierung von GNL1 zu dessen Loslösung vom prä-60S-Partikel im Cytoplasma führt. Bestätigen konnten wir dies insofern, als GNL1 nach Depletierung von CK2β, der strukturellen CK2-Untereinheit, in Sucrose-Gradienten vermehrt mit 60S-Untereinheiten assoziiert war.


Zusammenfassend lässt sich sagen dass die TAP-Methode, kombiniert mit der induzierbaren Depletion von bestimmten Proteinen mittels shRNA, ein wertvolles Werkzeug für die Forschung in der humanen Biogenese von Ribosomen darstellt. Einerseits wurde dadurch die Identifizierung von neuen Faktoren in diesem Prozess möglich, andererseits diente die Methode als Ausgangspunkt zur Erforschung einzelner Schritte in der Biogenese von Ribosomen.
1. Introduction

1.1 Ribosomes and their evolution

Ribosomes translate the genetic information into proteins, which represent most of the functional entities in living cells. Thus, ribosomes exert a key function in all organisms, from the simplest prokaryotes to metazoans. Ribosomes are built up of two parts, the large (LSU) and small (SSU) subunit. Each subunit consists of one to three pieces of RNA and several proteins. As some of these proteins are found in all domains of life (Harris et al. 2003) and due to their similar structure (Wilson and Nierhaus 2003), it is apparent that ribosomes have evolved from one common ancestor.

Whereas most catalytic functions in cells are performed by proteins, the ribosome contains enzymatically active RNA. This contributed to the hypothesis of the ancient “RNA world” (Dworkin et al. 2003), where the RNA replicated itself by ribozymes (catalytically active RNA). In the billions of years since then, ribozymes have become less and less important. Ribosomes, the putative remnants of the RNA world, depend on additional molecules like ribosomal proteins, elongation and initiation factors and tRNA for their proper functioning. Thus, the complexity of ribosomes has greatly increased during evolution. Concomitantly, ribosome biogenesis became a complex process that involves several hundred factors in higher organisms (Wolf and Koonin 2007; Fournier et al. 2010).

This thesis should contribute to the understanding of the assembly of ribosomes in mammals. In the introduction, the biogenesis of bacterial ribosomes will be described first, followed by a summary of recent data from research in yeast and mammalian ribosome biogenesis. Molecular mechanisms of particular steps of LSU and SSU biogenesis and cross-talks between ribosome biogenesis and other cellular pathways will be a special focus.

1.2 Ribosome biogenesis in bacteria

In *E. coli*, ribosome biogenesis has been studied for decades. The ribosome of this bacterium consists, like in all present-day organisms, of a small (30S) and a large (50S) subunit. The SSU is composed of 21 ribosomal proteins and the 16S rRNA, the LSU of 34 ribosomal proteins and two rRNA pieces, 5S and 23S. *In vitro* studies first detailed an assembly map of 30S subunits (Traub and Nomura 1969; Mizushima and Nomura 1970; Held and Nomura 1973; Kresge et al. 2007). In these experiments, ribosomal proteins of the small subunit were sequentially added to purified rRNA, showing that binding of some ribosomal proteins
(primary binders) is necessary before others can assemble. In living cells, ribosome biogenesis starts with transcription of a large rRNA containing the eventual mature rRNA sequences as well as internal transcribed spacers (ITS) in between. Presumably already during transcription, processing of the rRNA (cleavage and modification) and assembly of ribosomal proteins takes place (Kaczanowska and Ryden-Aulin 2007; Williamson 2008; Connolly and Culver 2009).

In addition to biochemical experiments, structural investigations contributed successfully to the understanding of ribosomes and ribosome biogenesis. Crystallization of the bacterial ribosome was possible at high resolution (Ban et al. 2000; Wimberly et al. 2000; Schuwirth et al. 2005). Many studies in ribosome biogenesis also in eukaryotes therefore rely heavily on these structures, as for eukaryotic ribosomes, crystallization was not yet possible.

1.2.1 The energy landscape of rRNA folding

Seminal experiments by Masayaso Nomura and Co-Workers around 1970 on assembly of the small subunit were based on incomplete folding intermediates at equilibrium. According to these experiments, a 21S assembly intermediate is formed below 15 °C, which changes conformation upon heating to 40 °C and then sediments at 26S. Assembly of late binding ribosomal proteins at again low temperatures yields the mature 30S particle.

![Assembly map of the 30S subunit](image)

**Fig. 1.1. Assembly map of the 30S subunit.**

Ribosomal proteins can be divided into two groups: primary binders attach to the 5’ end of the 16S rRNA first, followed by the secondary binders in the central and 3’ domain. Color codes depict binding rates, where known. Red are the fastest binders, followed by yellow, green, blue and purple. The blue ring around S5 represents the slower binding of the deacetylated form. Adapted from (Talkington et al. 2005).

Later experiments investigated the kinetics of binding of proteins to ribosomal subunits using mass spectrometric methods (Talkington et al. 2005). Early binders like Rps4 (see fig. 1.1) showed a much faster association rate than late binders like Rps12. However, the characteristics of binding are similar for all ribosomal proteins, and the existence of a 21S
intermediate is denied in this study. Instead, it is proposed that the *in vitro* assembly of the ribosomal subunit can follow different pathways through an energy landscape, which changes with every conformational change of the rRNA and every additional ribosomal protein bound to the complex (fig. 1.2).

**Fig. 1.2 Model of the changes of the energy landscape during assembly of ribosomal proteins.** The drawing represents the energy levels of different rRNA folding states. If a ribosomal protein (red and green sphere) binds to the rRNA, the landscapes changes such that the pathways leading to the eventual correct folding state are energetically favoured. Adapted from (Talkington et al. 2005).

The large 50S subunit can also be reconstituted *in vitro*, although the assembly map is obviously more complicated due to the larger number of ribosomal proteins (Rohl and Nierhaus 1982). As for the SSU, assembly seems to begin at the 5′ end of the 23S rRNA. The assembly of a group of proteins that are also necessary for a minimal peptidyltransferase activity occurs simultaneously. A second group of proteins was shown to be required for 5S rRNA binding. Interestingly, the genes for proteins of the same assembly cluster are grouped together in the *E. coli* genome.

### 1.2.2 Assembly of ribosomal subunits in vivo

Transcription of rRNA, the beginning of the ribosome biogenesis, makes up the most important part of all transcription activity in the cell, as mature rRNAs are among the most abundant RNA molecules in the cell. Because the levels of ribosomal proteins are controlled
by the amount of binding sites present at nascent rRNA molecules, the rRNA transcription is rate limiting for the assembly of new ribosomes (Paul et al. 2004). In *E. coli*, there are seven rRNA operons, which contain the 16S, 23S and 5S rRNA, and between the 16S and the 23S rRNA one to three tRNA genes (fig. 1.3). Transcription of the operon by the RNA polymerase is tightly regulated in response to changes in the environment and the growth phase of the bacterial culture. While the rRNA synthesis is low during stationary phase, addition of fresh medium leads to a quick upshift of transcription.

**Fig. 1.3 The *E. coli* rrnB operon.** Scheme of the transcript from this operon. P1, P2 and T1, T2 are promoters and terminators for the RNA polymerase, respectively. Sites of endonucleolytic cleavage are denoted by “x”. Adapted from (Kaczanowska and Ryden-Aulin 2007).

Initiation of transcription is regulated by various proteins, but interestingly also by nucleotides, especially by guanosine tetraphosphate ppGpp (two phosphates each at the 3’ and 5’ oxygens of the ribose). This nucleotide is produced upon starvation from ATP and GTP by the enzymes RelA and SpoT (Magnusson et al. 2005), and inhibits the RNA polymerase directly. As the first nucleotide needed for transcription in six of the seven rRNA operon is an ATP, transcription initiation relies heavily on this nucleotide, whose concentration decreases under unfavorable conditions (poor medium, stationary phase). Studies in *B. subtilis* showed many similarities to the rRNA transcription regulation in *E. coli*. However, the differences like distinct roles of GTP and ppGpp suggest that among prokaryotes many ways of rRNA transcription regulation exist.

Processing of the rRNA and assembly of ribosomal protein begins already during transcription (Connolly and Culver 2009). About 30 to 40 non-ribosomal proteins are involved in ribosome assembly, primarily in rRNA cleavage and modification. Interestingly,
most of the ribosome biogenesis factors in *E. coli* are not essential. This contrasts with the vital importance of ribosomes for the cells and indicates redundant or spontaneous processes. Most of the rRNA modifications involve methylation of riboses, whereas pseudo-uridylation is more rare. They occur mostly at sites important for translational events and may contribute to proper folding of the rRNA. However, some modification sites have been shown to be dispensable for assembly and function of ribosomes *in vitro* (Green and Noller 1996). Seven methyltransferases and one pseudouridine synthetase have been identified up to date in *E. coli*. Whereas most of the modification sites are not conserved throughout evolution, a double dimethylation site at the 3’ end of the 16S/18S rRNA exists both in prokaryotes and in eukaryotes. KsgA, related to Dim1, is the enzyme that is thought to carry out this modification. The release of KsgA is necessary for progression of the SSU biogenesis.

An evolutionary conserved role, like the one found for KsgA/Dim1, may as well be exerted by a family of circularly permutated GTPases. The release of a member of this family in *E. coli*, YlqF, is necessary for further maturation steps. In eukaryotes, Lsg1 and homologues thereof are also involved in LSU processing.

**1.3 Ribosome biogenesis in yeast**

Compartmentalization is a hallmark of eukaryotes. One of these compartments is the nucleus, which does not exist in prokaryotes. This leads to additional complexity in the process of ribosome biogenesis, as ribosomal proteins have to be imported into the nucleus, and the nascent subunits exported into the cytoplasm (fig. 1.4).
**Fig. 1.4. Overview on eukaryotic ribosome synthesis.** After transcription of the rRNA, ribosomal proteins and trans-acting factors associate to form a 90S particle. Cleavage of the pre-rRNA leads to a pre-40S and a pre-60S particle that are processed in the nucleolus und nucleus. After export to the cytoplasm, final maturation steps yield ribosomal subunits ready for translation.

From prokaryotes to eukaryotes, complexity further increased due to the higher number of ribosomal proteins. In *S. cerevisiae*, there are 32 ribosomal proteins for the small subunit and 46 ribosomal proteins for the large subunit (compared to 21/34 for *E. coli*), and four instead of three molecules of rRNA (18S; 5S, 5.8S and 25S) can be found. Moreover, ribosome assembly relies on more than 170 trans-acting factors and around 70 snoRNAs (Fromont-Racine et al. 2003; Tschochner and Hurt 2003; Zemp and Kutay 2007; Henras et al. 2008; Kressler et al. 2009; Strunk and Karbstein 2009). A cryo electron microscopy map structure for a eukaryotic ribosome is available (Spahn et al. 2001), however presumably lacking some ribosomal proteins. Still, as described later in two cases, also low-resolution images can contribute to the elucidation of molecular mechanisms of trans-acting factors in ribosome biogenesis (see section 1.3.3).

An interesting point is that these trans-acting factors are mostly essential in yeast, in contrast to their counterparts in *E. coli*. A reason could be differences in rRNA folding. Current hypothesis suggest that the biogenesis of ribosomes in prokaryotes occurs through an “assembly gradient”, where binding of ribosomal proteins is coupled to the folding of the rRNA (Dinman 2009). In eukaryotes, however, this process might require much more chaperoning by proteins supporting the folding.

**1.3.1 Transcription of ribosomal RNA**

A large 35S pre-rRNA, consisting of the 18S, 28S and 5.8S rRNA and intergenic spacers is transcribed in the nucleoli, which form around the nucleolus organizing regions (NOR) that surround the rDNA repeats. Not only the principle of a large pre-rRNA transcript is like in *E. coli*, there are also several rDNA repeats, similar to the rRNA operons in prokaryotes. The rRNA is transcribed by RNA polymerase I, one of the three RNA polymerases. Transcription starts with an initiation complex comprising several proteins (fig. 1.5) and depends on the presence of Hmo1p (UBF in *H. sapiens*) at the rDNA. This protein is only found in active rDNA loci.
In each NOR, there are hundreds of rDNA repeats. But even in rapidly growing cells, only about half of all repeats are actually transcribed. A downshift in rRNA production, for example after entry into stationary phase, whereas rDNA transcription is reduced about 10 times, seems to occur both through the regulation of the transcription machinery and through the number of active genes (Moss 2004).

**Fig. 1.5. Polymerase I initiation complex.**

Active rDNA loci are characterized by the presence of Hmo1p/UBF. At these sites, the transcription initiation complex is formed. After binding of RNA Polymerase I multimer via Rrn3, transcription can start. Adapted from (Moss 2004)

### 1.3.2 Processing and modification of the rRNA

Pre-rRNA is transcribed by RNA polymerase I as a primary 35S transcript, which then undergoes several processing steps (fig. 1.6). Separately, RNA polymerase III transcribes the 5S rRNA, which is afterwards incorporated into nascent 60S subunits in complex with the ribosomal proteins L5 and L11 (Steitz et al. 1988; Zhang et al. 2007). In contrast to the mechanisms of *trans*-acting factors in ribosome biogenesis (see section 1.3.3), processing of the rRNA is rather well studied. This processing begins already during transcription in a coordinated manner (Gallagher et al. 2004; Osheim et al. 2004). Most of the cleavage events occur in the nucleolus and result in the production of 20S rRNA, an 18S rRNA precursor, 7S rRNA, a precursor of the 5.8S rRNA, and mature 25S rRNA.

The pre-40S particle, containing the 20S pre-rRNA is released into the nucleoplasm and then exported to the cytoplasm. There, final maturation occurs by cleavage of the rRNA at site D by the endonuclease Nob1p (Fatica et al. 2003; Fatica et al. 2004; Lamanna and Karbstein...
The 7S rRNA is processed in the nucleus to 6S pre-rRNA by Rrp6 and finally matured to 5.8S rRNA in the cytoplasm by Ngl2 (Thomson and Tollervey 2010).

Apart from endo- and exonucleases, around 70 different small nucleolar RNAs (snoRNAs) participate in rRNA processing and modification. SnoRNAs do not exist in prokaryotes; however, they can be found in Archaea. The snoRNA molecules associate with a number of proteins in two classes of snoRNPs, named box C/D and box H/ACA. Box C/D snoRNPs consist of Nop1p, Nop56p, Nop58p and Snu13p and one RNA molecule of about 100 nt. Box H/ACA are built up of a 200 nt RNA and the proteins Nhp2p, Nop10p, Gar1p and Cbf5p.
Fig. 1.6 Overview of the rRNA processing pathway. The RNA Polymerase I transcribes the primary transcript shown in (A). Several cleavage steps (B) then lead to the mature rRNA. Note that for some parts alternative pathways exist. Adapted from (Poll et al. 2009).

As every 35S pre-rRNA associates with about 70 snoRNPs, this would give a complex of about 17 MDa, compared with 1-2 MDa of mature ribosomal subunits, when all snoRNPs bind simultaneously (Hage and Tollervey 2004). However, it is not clear whether this is indeed the case or whether the snoRNPs bind in a different manner like sequentially or in modules. Because snoRNAs bind often to regions of the rRNA that are tightly packed in the mature structure, it is thought that binding of many snoRNPs occurs co-transcriptionally. However, as the number of snoRNPs is lower than that of nascent rRNA molecules, there might be other mechanisms that prevent folding of the pre-rRNA unless the snoRNAs bind. The two classes of snoRNPs carry out different modifications. Box C/D snoRNPs are responsible for 2'-O-methylation of riboses, whereas box H/ACA snoRNPs target sites of pseudo-uridylation. Up to now, 53 methylation sites and 45 pseudo-uridines have been identified (Decatur et al. 2007). Some snoRNAs are also involved in rRNA cleavage. The snoRNA targets the complex to the proper position on the pre-rRNA by complementary base pairing and the associated proteins mediate the modifications. Modification of the rRNA does not always rely on snoRNPs. As mentioned in section 1.2.2, a conserved adjacent dimethylation can be found in the rRNA of the small ribosomal subunit. In yeast, this methylation is carried out by the protein Dim1p (Lafontaine et al. 1994; Lafontaine et al. 1995; Lafontaine et al. 1998). Later investigations showed that Dim2p is necessary for Dim1p to carry out this function (Vanrobays et al. 2004). However, the exact function of this and many other rRNA modifications is still unknown.

1.3.3 Molecular mechanisms in ribosome biogenesis

Recent years brought significant progress in the identification of the factors involved in yeast ribosome biogenesis. Different pre-ribosomal particles of both subunits were isolated and the components identified by mass spectrometry and Western blotting (Bassler et al. 2001; Harnpicharnchai et al. 2001; Dragon et al. 2002; Nissan et al. 2002; Schafer et al. 2003; Oeffinger et al. 2007). On one hand, these studies allowed an overview of the proteins involved in ribosome biogenesis. On the other hand, staging of particles and trans-acting factors was possible to a certain extent. This was a pre-requisite for further investigations into
molecular mechanisms. However, to date only a very limited number of such mechanisms have been described. Five of them will be summarized in this section.

**Kinase-mediated remodeling of pre-40S subunits and final 18S rRNA maturation**

Mature 40S subunits contain a protrusion of helix 33 in the 18S rRNA visible in electron microscopy. This protrusion was shown to be formed after phosphorylation of Enp1 and Rps3 by the kinase Hrr25 (Schafer et al. 2006). Firstly, it was observed that Ltv1, Enp1 and Rps3 form a salt stable complex and that all three proteins seem to be phosphorylated. Rio2 and Hrr25 (a casein kinase I isoform involved in different cellular processes), both part of pre-40S particles, where candidate kinases for these phosphorylations. In cells depleted of Hrr25, Enp1 and Rps3 were no longer phosphorylated and not released from pre-40S particles. Ltv1 was only partly phosphorylated. Hence, all three proteins were thought to be substrates of Hrr25. Addition of ATP led to dissociation of the trimeric complex, which seems odd, as Rps3 needs to stay associated to the pre-40S particle in the course of 40S biogenesis. Only a subsequent dephosphorylation step yielded a salt-stable association of Rps3 to the pre-ribosomal subunit. As no phosphatase is known to act in ribosome biogenesis, the authors used \( \lambda \) phosphatase for this experiment. After phosphatase treatment, a subpopulation of all Rps3 eventually associated with the 40S particles also at 100 mM MgCl\(_2\).

These *in vitro* experiments were confirmed by electron microscopy imaging. After the phosphorylation-dephosphorylation cycle, the protrusion of helix 33 appeared. Assembly of Rps3 to the nascent 40S subunit therefore seems crucial for this structural rearrangement.

Pre-40S particles exported to the cytoplasm contain 20S pre-RNA. After export of the particle to the cytoplasm, the rRNA undergoes final processing. The endonucleolytic cleavage is thought to be performed by Nob1p (Fatica et al. 2003; Fatica et al. 2004) and also requires Fap7 (Granneman et al. 2005). In two recent studies, the mechanism was explored further (Lamanna and Karbstein 2009; Pertschy et al. 2009). Firstly, two additional proteins, the helicase Prp43 and its cofactor Pfa1 were shown to be involved in the cleavage step. Overexpression of Nob1 suppressed the slow growth phenotype caused by the co-knockout of Prp43/Ltv1 or Pfa1/Nob1, indicating that they are necessary for efficient processing. An artificial RNA substrate containing the stem loop around the cleavage site D (see fig. 1.6) was cleaved by purified Nob1, but only in an inefficient manner in presence of manganese.
Remodeling of a nuclear 60S precursor

For the pre-40S subunit, a remodeling seems to occur following Hrr25 phosphorylation (described above). Also for the large subunit, such a remodeling was described (Ulbrich et al. 2009). Rea1, a gigantic ATPase of about 550 kDa was found to be associated to pre-60S particles in the nucleus (Galani et al. 2004; Nissan et al. 2004). Rea1 is attached to the pre-60S particle through a trimeric subcomplex consisting of Ipi3 and Rix1 at the bottom of the pre-ribosomal subunit. At the end of a flexible, elongated part of Rea1, a MIDAS domain interacts with Rsa4, which is located at the side of the pre-60S particle, as detected by electron microscopy. When Rea1 binds to Rsa4, its ATPase activity is triggered, which leads to dissociation of Rsa4, Ipi3, Rix1 and Rea1 from the subunit. This step is thought to make the pre-60S particle competent for export to the cytoplasm.

Cytoplasmic steps in 60S subunit maturation

After export of the ribosomal subunits to the cytoplasm, proteins involved in export (see section 1.3.4 for details) need to be removed from the nascent subunits. Arlen Johnson and co-workers looked into the removal of the putative 60S export factor Nmd3 from cytoplasmic pre-60S particles (Kallstrom et al. 2003; Hedges et al. 2005; West et al. 2005). Genetic and biochemical experiments were used to show that the GTPase Lsg1 is involved in the release of Nmd3. This enzyme catalyzes both the release of Nmd3 and the loading of the ribosomal protein Rpl10. The WD40 protein Sqt1 is thought to chaperone Rpl10, and they join onto the pre-60S particles coming out of the nucleus as a heterodimer. GTP hydrolysis by Lsg1 would thus trigger the release Nmd3, Sqt1 and also Lsg1 from the particle.

Another step in the cytoplasmic maturation of the large ribosomal subunit is the assembly of the ribosomal stalk. This protrusion consists of the Rpp proteins, distinguished from the ribosomal proteins by their negative charge, namely P0 and two copies of P1 and P2 each (Ballesta and Remacha 1996). Mrt4, a protein that localizes mostly at the nucleolus at steady state, shows considerable homology to P0, which is in contrast to Mrt4 exclusively cytoplasmic. Based on this observation, it was suggested that Mrt4 exchanges for P0 in the cytoplasm (Kemmler et al. 2009; Lo et al. 2009; Rodriguez-Mateos et al. 2009a; Rodriguez-Mateos et al. 2009b). The phosphatase Yvh1 was shown to be necessary for the release of Mrt4 from the pre-60S subunits, and an Mrt4 mutant with reduced affinity to the particle bypassed the need of Yvh1 for the exchange of Mrt4 for P0. Also Rpl12, localized next to P0
at the base of the stalk, was necessary for the process. Other proteins involved in late 60S biogenesis like Lsg1 or Efl1 seemed not to be required for Mrt4 recycling.

Up to now, it is not clear to what extent events in cytoplasmic 60S maturation are coordinated and whether all factors are known (Panse and Johnson 2010). A functional network of trans-acting factors has been proposed (Lebreton et al. 2006), comprising the release of Tif6/Sdo1 by Efl1 (Senger et al. 2001; Menne et al. 2007), the action of Drg1 (Pertschy et al. 2007), and the exchange of Rpl24 for Rlp24 and release of Arx1/Alb1, which might require Rei1 (Parnell and Bass 2009). An overview of the processes known so far is given in fig. 1.7.

![Fig. 1.7. Exchange events on cytoplasmic pre-60S particles.](image)

**Fig. 1.7. Exchange events on cytoplasmic pre-60S particles.** Note that the order here does not imply a sequence of events happening in the cell.

### 1.3.4 Nuclear export of ribosomal subunits

In eukaryotes, the genome is enclosed in the nucleus and segregated from the cytoplasm by the nuclear membrane. Nuclear pore complexes (NPCs) mediate traffic across this membrane. All nascent ribosomal subunits – about 2000 per minute in rapidly growing yeast cells – have therefore to pass through these gates, which requires the assistance of an efficient export machinery.

Initially, the nuclear export of ribosomal subunits has been studied with GFP tagged ribosomal proteins (Hurt et al. 1999; Moy and Silver 1999; Stage-Zimmermann et al. 2000; Moy and Silver 2002). In steady state, these proteins and therefore the GFP signal is detected in the cytoplasm. When nuclear export of pre-ribosomal subunits is impaired, the GFP signal is relocalized to the nucleus.

This assay revealed that several nucleoporins (NPCs associated proteins) and transport receptors are necessary of the export of ribosomal subunits. Not only exportins but also importins were shown to be important for efficient export, which could mean that import of certain factors is a prerequisite of the export of ribosomal subunits.
Following these initial studies, Crm1 was demonstrated to be necessary for export of both small and the large subunits (Ho et al. 2000; Gadal et al. 2001). Crm1 recognizes signal peptides in proteins, the so called nuclear export signals (NES) characterized by several hydrophobic residues (Kutay and Guttinger 2005). For 60S subunits, Nmd3 was suggested to act as an NES containing export adaptor and to shuttle in and out of the nucleus in a Crm1-dependent manner. At the C-terminus of Nmd3, there is an NES whose deletion leads to a block in 60S export. Although a trimeric complex of 60S subunits together with Crm1 and Nmd3 could not be reconstructed in vitro, no biochemical data contesting the hypothesis of Nmd3 as an export factor have been published so far.

In addition to Nmd3/Crm1, other proteins have been identified to be involved in the export of 60S ribosomal subunits. The dimer Mex67/Mtr2 was originally shown to be necessary for mRNA export (Segref et al. 1997; Santos-Rosa et al. 1998). Later, the two proteins have also been associated with pre-60S export (Bassler et al. 2001; Nissan et al. 2002; Yao et al. 2007). Interestingly, it was possible to generate alleles that showed deficiencies in 60S but not in mRNA export. It was then proposed that Mex67/Mtr2 play a role in the coordination of mRNA export and ribosome biogenesis (Yao et al. 2008).

Another 60S trans-acting factor, Arx1, was shown to bind nucleoporins directly without any interaction with transport receptors (Bradatsch et al. 2007; Hung et al. 2008). The 3D structure of the human homologue of Arx1 showed that Arx1 adopts the fold of a methionine aminopeptidase, however does not display such activity in vitro. Still, the active site of Arx1 may be at the same location where it would be in an aminopeptidase. Deletion of Arx1 alone did not impair 60S export, but the sensitivity for mutations or deletion of other proteins involved in export was increased. For example, alleles of Mtr2 or Mex67 that would not lead to growth defects in wild type cells, turned out to be lethal when Arx1 was deleted.

A similar role as for Arx1 was described for Rrp12 (Oeffinger et al. 2004) and recently for Ecm1 (Yao et al. 2010). The HEAT repeat protein Rrp12 is unusual in that it interacts with both the small and the large subunit. In summary, it seems that various factors are directly involved in the export of the large ribosomal subunit.

For the small subunit, the process of nuclear export is less studied. Three proteins, Dim2p, Ltv1p and the kinase Rio2p, are currently the proposed candidates for Crm1 adaptors for the small ribosomal subunit (Vanrobays et al. 2003; Seiser et al. 2006; Vanrobays et al. 2008; Fassio et al. 2010). However, how directly these proteins contribute to 40S export is still under investigation.
1.4 Ribosome biogenesis in higher eukaryotes

For a long time, the principal model organisms for the study of ribosome biogenesis have been *E. coli*, *B. subtilis* and *S. cerevisiae* because they are genetically amenable. In *S. cerevisiae* also proteomic approaches like the TAP library contributed to the progress in this field. In comparison, ribosome biogenesis is less well investigated in mammalian cells. Studying ribosome biogenesis in higher eukaryotes may therefore yield valuable additional insights like the identification of factors involved in this pathway not present in yeast or the elucidation of different mechanisms. One example is the protein nucleophosmin, which is absent in yeast (Lim and Wang 2006; Frehlick et al. 2007). In addition to other functions in the cell, described in more detail in section 1.5, nucleophosmin carries out an early rRNA cleavage step.

The primary and mature rRNA transcripts and the ribosomal proteins in human cell lines are well known. Compared to yeast, the number of ribosomal proteins in mammalian cells has increased to 34 from 32 for the small subunit, and to 48 from 46 for the large subunit. The largest rRNA in human cells is 28S compared to 25S in yeast. Interestingly, the primary transcript is much longer than this slight increase insinuates. Whereas the primary RNA polymerase I transcript is a 35S molecule in yeast, it is a 47S molecule in human cells. The spacers between the actual rRNA moieties are therefore much larger, but whether they fulfill additional roles has not yet been studied.

A structure of the mammalian ribosome at atomic resolution is still out of reach. Recently, a cryo-electron microscopy structure of a mammalian ribosome was published (Chandramouli et al. 2008). The resolution of 8.7 Å allowed conclusions on the overall structure.

As different names for the proteins described here can be found in the literature, tab 2.1. summarizes the terms used here and the respective database names and yeast homologues.

1.4.1 Transcription, modification and processing of mammalian rRNA

In the human genome, several hundred rDNA repeats are distributed on five different chromosomes (Henderson et al. 1972; Grummt 2003; Drygin et al. 2010). The rRNA coding region is about 14 kb long, with intergenic regions of about 30 kb in between. Upstream of the promoter, repetitive enhancer elements are found. The rRNA is transcribed by the RNA polymerase I only from active rDNA loci, which are characterized by the binding of UBF (fig. 1.5).

During each cell generation, about 1 to 2 millions of ribosomes are synthesized. Transcription of rRNA, where ribosome biogenesis begins, is controlled by regulation of the transcription
factors and long-term mechanisms like silencing of genes. In addition to histone deacetylation, also constitutive long-term silencing of rDNA loci exists in higher eukaryotes and plants, a mechanism presumably based on DNA methylation (Moss 2004).

Like in yeast, snoRNPs can be found in mammals, which modify about twice as many nucleotides as in yeast (200 instead of 100) (Decatur et al. 2007). Interestingly, most of the snoRNAs are encoded by introns of proteins involved in ribosome biogenesis (Yang et al. 2006).

The processing pathway of human rRNA is basically similar to the yeast counterpart (fig. 1.8). However, the scheme is still very undeveloped (compare fig. 1.8 with fig. 1.4), and many players of specific rRNA processing and modification steps are not yet identified. Furthermore, new precursors or alternative pathways may show up (Rouquette et al. 2005).

Fig. 1.8. Processing of the rRNA in human cells. The RNA Polymerase generates a 45S/47S primary transcript, which is about one third larger than in yeast cells. The overview is only presented schematically, as most proteins involved in the various processing steps are unknown. Note that the final 18S rRNA processing shows an additional species (18SE) compared to yeast. From (Henras et al. 2008).

1.4.2 Maturation of ribosomal subunits

Up to date, only two pre-ribosomal particles of mammalian origin have been described (Fujiyama et al. 2002; Zemp et al. 2009). Knowledge about pre-ribosomal particles in higher eukaryotes is therefore mostly based on the assumption that the particles are conserved through evolution. This was indeed confirmed in these two studies, however, also previously unknown proteins were detected. Likewise, a microscopy-based RNAi screen in HeLa cells with various reporters for 40S and 60S maturation confirmed the involvement of proteins homologous to yeast trans-acting factors but also revealed an involvement of novel proteins.
(T. Wild et al., manuscript in preparation and T. Wild, unpublished results). In this section, the current knowledge about pre-ribosomal factors in human will be described.

Seminal studies of ribosomal subunit maturation in metazoans, namely the nuclear export, were published two decades ago, ten years before the corresponding experiments in yeast. Injection of purified, radioactively labeled subunits into X. laevis oocytes showed a unidirectional and saturable process requiring energy (Khanna-Gupta and Ware 1989; Bataille et al. 1990). The authors tested subunits of four different organisms including prokaryotic E. coli ribosomal subunits. Subunits were exported from the nucleus, but not imported when injected into the cytoplasm.

**Cytoplasmic 40S maturation**

Rio2 is a kinase involved in a late cytoplasmic step of 40S biogenesis. In yeast, it has been shown to be involved in cytoplasmic 20S processing (Geerlings et al. 2003; Vanrobays et al. 2003) and may play a role in 40S export (see also section 1.2.). In human cells, late pre-18S RNA processing contains an additional step compared to yeast. Mature 18S rRNA is produced from 21S rRNA via an 18S-E intermediate. It is this very last processing step that depends on Rio2 in human cells (Rouquette et al. 2005; Zemp et al. 2009). In addition to the requirement in rRNA processing, it was shown that Rio2 contains an NES that contributes to 40S export. Furthermore, human Rio2 is required for the release of different 40S trans-acting factors from cytoplasmic pre-ribosomal particles and hence their subsequent recycling back to the nucleus. Whereas Enp1 recycling to the nucleus only needed the physical presence of Rio2, the three proteins Dim2, Ltv1 and Nob1 could not be recycled to the nucleus, when endogenous Rio2 was depleted and a kinase dead variant of Rio2 was overexpressed. Taken together, the data indicates that more than one step occurs in the cytoplasmic 40S maturation in human cells (Zemp et al. 2009). The proteins that are phosphorylated by Rio2 are however still under investigation.

**Nuclear export of pre-ribosomal subunits**

Also human cells rely on the Crm1 pathway to export ribosomal subunits, as export of both subunits is blocked upon inhibition of Crm1 (Thomas and Kutay 2003). In this publication, a crucial role for Nmd3 for pre-60S was shown. Hence, the export adapter function of this protein seems to be conserved, as it is also crucial for export of the 60S subunits in yeast (Thomas and Kutay 2003; Trotta et al. 2003).
Like the pre-60 particles, also the pre-40S ribosomal subunits rely on Crm1 for nuclear export. The kinase Rio2 (described above) binds directly to Crm1 in a Ran dependent manner (Zemp et al. 2009). However, depletion of Rio2 only reduced but did not block 40S export, indicating that other Crm1 adaptors or other export receptors are involved.

Whereas the role of Nmd3 seems to be conserved throughout evolution, this does not seem to be the case for Mtr2/Mex67 (see section 1.3.4). Depletion of the human homologues, p15 and TAP, in HeLa cells does not impair export of pre-60S subunits. Instead, the role of Mtr2/Mex67 may be fulfilled by XPO5, which presumably binds directly to RNA (T. Wild, manuscript in preparation). XPO5 was previously shown to export microRNAs from the nucleus to the cytoplasm (Lund et al. 2004). Also the human homologue of Arx1p, described in section 1.3.4, termed Ebp1, does not seem to participate in nuclear export of pre-ribosomes. Whereas Arx1p associates with FG repeat nucleoporins, Ebp1 does not (Bradatsch et al. 2007). Ebp1 was crystallized and shown to interact with RNA (Kowalinski et al. 2007). Furthermore, it might influence translation (Squatrito et al. 2006) and transcription, and interacts with the EGF receptor ErbB3 (Hamburger 2008). It has also been shown to interact with pre-ribosomes and ribosomes (Squatrito et al. 2004; Kowalinski et al. 2007). Taken together, the data so far published about yeast and human Arx1 shows that the human protein has acquired additional functions and maybe lost its property as an export adaptor during evolution.

Conserved and new factors in 60S maturation in human cells

Only very few studies about mammalian pre-60S maturation have been published so far. The knowledge therefore remains fragmentary, and mostly relies on data from experiments in yeast. In this part, four proteins will be described. Rex4 and Lsg1 are examples for protein families, which have, in comparison to the yeast genome, additional members in higher eukaryotes. Wdr12 and Tif6, on the other hand were shown to have gained additional roles in human cells, similar to Arx1 described above. These two developments might be discovered for more pre-ribosomal trans-acting factors in future research.

In the human genome, the number of genes is much larger compared to the yeast genome. Accordingly, some yeast proteins have more than one human orthologue. This was observed for the Rex4 family of exonucleases. In the human genome, four variants of this protein can be found, Rex4, Isg20, Isg20L1 and Isg20L2 (Coute et al. 2008). This increase in trans-acting factors could indicate an increased complexity of the processes or more layers of regulation.
Whereas the exonuclease activity of the human proteins was confirmed, no further insights were presented in this publication.

A similar conclusion concerning the augmentation of trans-acting factors could be drawn from a study on the Lsg1 family of GTPases (see also section 1.3.3). This family contains three members in yeast, whereas four homologues can be found in D. melanogaster and five in humans (Reynaud et al. 2005).

The WD40 repeat protein Wdr12 was found to localize in the nucleolus (Holzel et al. 2005) like its yeast counterpart Ytm1p (Huh et al. 2003). The 32S rRNA precursor was enriched after Wdr12 RNAi and simultaneously the 28S rRNA was reduced, indicating a role of Wdr12 in 60S biogenesis. Like in yeast, Wdr12 forms a complex with two other proteins, Pes1 (Nop7p) and Bop1 (Erb1p), which are also required for proper rRNA processing. But Wdr12 is also interconnected with other pathways: its expression seems to be regulated by c-myc, and overexpression of a truncated Wdr12 construct increased p53 levels (Holzel et al. 2005; Holzel et al. 2007; Rohrmoser et al. 2007).

The pre-60S factors Tif6 seems to perform multiple tasks. It was first described as an anti-association factor, hindering the small and large ribosomal subunits to join (Russell and Spremulli 1979; Valenzuela et al. 1982). Later, it has also been shown that Tif6 is required for 60S biogenesis in yeast (Basu et al. 2001; Senger et al. 2001). Like Enp1/Ltv1/Rps3 (see section 1.3.3), Tif6p seems to be phosphorylated by the casein kinase I homologue Hrr25p (Ray et al. 2008). This phosphorylation is necessary for the progress of 60S biogenesis.

In human cells, Tif6 acts also in translation (Miluzio et al. 2009). Only small amounts of Tif6 may be needed for ribosome biogenesis, acting together with SDBS. As far as its role in translation is concerned, Tif6 might be involved in miRNA-mediated translation repression. Release of Tif6 from a pre-60S or mature 60S particle may be phosphorylation-dependent and/or mediated by the GTPase Efl1p (Senger et al. 2001). Tif6 presumably binds to the 60S subunits via Rpl23 (Benelli et al. 2009; Gartmann et al. 2010)

### 1.5 Regulation of ribosome biogenesis and connections to other pathways

Ribosome biogenesis may be one of the most energy consuming processes in living cells. Cells are capable to synthesize up to 2000 ribosomes per minute, which means transcription of rRNA and ribosomal mRNA, processing and maturation, translation of ribosomal proteins and trans-acting factors and their import into the nucleus, and nuclear export – all energy consuming processes that are closely linked to growth and proliferation. Therefore, the
process of ribosome biogenesis needs to be tightly regulated at different levels. Results from research in both yeast and human will be summarized in the following sections.

1.5.1 Regulation of rRNA transcription

Yeast cells quickly sense changes in the environment. When conditions become unfavorable for further growth, signals are relayed to the nucleus to stop rRNA transcription. An important part of this communication is performed by the TOR pathway (Wullschleger et al. 2006) (fig. 1.9). At the heart of this pathway stands the TOR kinase complex, comprised of the TOR kinase and several other proteins. In yeast, two different complexes can be found, TORC1 and TORC2. TORC2 acts on actin organization, and will not be described here. The mammalian counterparts are mTORC1 and mTORC2. As the pathway in yeast and mammals seems to be quite similar, results from both organisms will be summarized here.

The TOR complex binds directly to rDNA, where it acts in two ways. One target is UBF/Hmo1p. UBF binds to the rDNA and activates transcription. TOR works indirectly on UBF via the RSK kinase family (Lempiainen and Shore 2009). The second target is a key coactivator of the RNA polymerase I, TIF-1A/Rrn3p. Upon phosphorylation, this protein interacts with the Rpa43 subunit of the RNA polymerase complex to promote transcription (Mayer et al. 2004).

TOR not only influences rRNA transcription, but also transcription of the mRNAs of ribosomal proteins and trans-acting factors. TORC1 phosphorylates Sfp1p, which regulates the gene transcription together with Ifh1. Interestingly, the phosphorylation of Sfp1p, necessary for its nuclear localization, seems to be unaffected by osmotic stress and starvation.

![Fig. 1.9. Overview of the RNA polymerase regulation by TOR in S. cerevisiae.](image-url)

The upper part shows organization of the promoter complexes under optimal conditions, the lower part
upon growth inhibition, which occurs after reduction of the activity of the TOR complex. Adapted from (Lempiainen and Shore 2009).

Starvation is not only sensed by TOR. Another pathway, independent of TOR, relies on the NAD\(^+\)/NADH ratio, which is increased when the ATP levels are reduced. By mediation of eNoSC (energy-dependent nucleolar silencing complex), this leads to methylation of rDNA and deacetylation of histones, thereby silencing the rDNA loci. The eNoSC contains three proteins, nucleomethylin (NML), SIRT1 and SUV39H1. NML binds to dimethylated histone H3, whereas SIRT1 is a NAD\(^+\)-dependent histone deacetylase. Finally, SUV39H1, a methyltransferase involved in heterochromatin formation, is activated by SIRT1 under conditions of low cellular ATP concentration and increased NAD\(^+\)/NADH ratio (Lempiainen and Shore 2009).

**Regulation of rRNA transcription by kinase cascades**

UBF is not only phosphorylated via TOR, as mentioned above, but also by G1 and S phase specific kinases (Drygin et al. 2010). Additionally, the interaction between UBF and SL1, a protein complex that is part of the transcription initiation complex (fig. 1.5), is impaired by phosphorylation of an SL1 component by Cdk1, also lowering rRNA transcription in mitosis. Also the ERK kinase, stimulated by mitogenic signals, is involved in regulation of rRNA transcription. On one hand, it acts via UBF phosphorylation. On the other, it also regulates the interaction between TIF-1A/Rrn3p and the Rpa43 subunit of the RNA polymerase directly or via RSK, like TOR (Drygin et al. 2010).

A third kinase cascade affecting ribosome biogenesis besides TOR and ERK involves the casein kinase II (CK2). CK2 promotes cell cycle progression and growth. Several proteins involved in rRNA transcription and ribosome biogenesis, like UBF, TIF-1A, SL1, nucleophosmin and nucleolin, are phosphorylated by CK2. In general, this leads to an increased synthesis of ribosomal subunits.

Further regulation of rRNA transcription, and also coordination of all three RNA polymerases, may involve the protein c-myc, an ubiquitous transcription factor and putative functional homologue of Sfp1. Beside promotion of transcription of other proteins, c-myc acts on transcription of UBF, ribosomal proteins and ribosome biogenesis trans-acting factor genes (Brown et al. 2008). It interacts also with rDNA and SL1. C-myc can be inhibited by the ribosomal protein Rpl11, whose mRNA transcription it in turn promotes, thereby constituting a negative feedback loop (Dai et al. 2007) (see also section 1.5.2 for the role of
Rpl11 in p53 regulation). Recently, also the promotion of RNA Polymerase III transcription of 5S rRNA and tRNA by c-myc was shown to be inhibited by Rpl11 (Dai et al. 2010). Apart from the short-term regulation of rRNA transcription, also long-term silencing of rDNA exists. Methylation of rDNA impairs UBF binding, thereby inhibiting transcription (McStay and Grummt 2008). Acetylation and methylation of histones, controlled both by protein and non-coding RNA, regulates the transcriptional state of the rDNA loci. Transcription of RNA from a site about 2 kb upstream of the pre-rRNA transcription start site has been shown to play an important role in heterochromatin formation and rDNA silencing.

1.5.2 Control of the p53 pathway by ribosomal proteins

The mechanisms described in the section before usually lead to upregulation of rRNA transcription. In response to various stimuli, cells begin to produce more ribosomes to enhance growth and proliferation. However, for most cells in multicellular organisms, growth is not a primordial task, and therefore ribosome biogenesis is tightly controlled. A central protein controlling cell growth is p53, which integrates and relays many inhibitory signals (Kruse and Gu 2009; Vousden and Prives 2009). It is therefore not surprising that p53 also acts on ribosome biogenesis. Synthesis of the rRNA is shut down in response to elevated p53 activity (Drygin et al. 2010), presumably by interaction of p53 with the members of the SL1 complex (fig. 1.5), thereby causing a transcriptional arrest. Similarly to p53, also tumor suppressors of the Rb protein family act on rRNA transcription.

Ribosome biogenesis is not only regulated at the level of rRNA transcription, but also during rRNA processing. The nuclease nucleophosmin is inhibited by p14ARF, a protein that stabilizes p53 by interfering with Mdm2, thereby impairing rRNA processing (Moss 2004). But p53 not only signals to the ribosome biogenesis pathway, it also senses when ribosome biogenesis is disturbed. Many stress signals are commonly processed by p53, and impaired ribosome biogenesis is one of them. This was for example seen when a mutant form of the nucleolar protein Bop1 was overexpressed, resulting in impaired 28S and 5.8S processing and p53 activation (Pestov et al. 2001). Recently, a similar effect was reported for WD3, a homologue of yeast UTP13p, part of the SSU processome. Like the overexpression of a mutant form of Bop1, depletion of WD3 lead to impaired 18S rRNA processing and elevated p53 levels (McMahon et al. 2010). Various ribosomal proteins seem to play an important role for p53 activation together with Mdm2 (Zhang and Lu 2009). Mdm2 is an E3 ubiquitin ligase that impairs p53 activity by sequestration and by funneling it into ubiquitin-dependent degradation by the proteasome.
Inactivation of Mdm2 is therefore important to upregulate the amount and activity of p53. Nucleolar stress induced by the transcription inhibitor actinomycin D or other effectors leads to binding of Rpl5, Rpl11 or Rpl23 and maybe also Rpl27L to Mdm2, which leads to inhibition of its ubiquitin ligase function. The three confirmed Mdm2 binders all attach to different parts of Mdm2 and are all necessary for full p53 activation, indicating non-redundant roles.

Rps7 seems to fulfill a more complex task, as it is also a ubiquitination substrate of Mdm2. At last, Rpl26 is also ubiquitinated by Mdm2 and serves as a translation activator for p53 by binding to the 5’ end of the p53 mRNA.

Not only ribosomal proteins may interact with p53 and/or Mdm2: So far, one trans-acting factor, GNL3, was also shown to bind p53 (Tsai and McKay 2002) and Mdm2 (Dai et al. 2008). GNL3/Nucleostemin is a member of the Lsg1 family of circularly permutated GTPases that are all implicated in 60S biogenesis in yeast.

Apart from their influence on p53, more and more extra-ribosomal functions of ribosomal proteins come to light (Warner and McIntosh 2009). For example Rpl23, already mentioned here as an Mdm2 inhibitor, also has a pro-proliferative role. The activation of Miz1, a transcription factor, by nucleophosmin leads to cell cycle arrest by triggering expression of the CDK inhibitors p15INK and p21CIP. Rpl23 sequesters nucleophosmin, thereby impairing the activation of Miz1. Thus, extra-ribosomal Rpl23 acts both in favor and against cell proliferation.

An interesting case is Rpl13a, which leaves the mature ribosome after stimulation of the cells by interferon-γ. Rpl13a then joins other proteins, amongst them a tRNA synthetase, to inhibit translation of certain mRNAs by binding to their 3’-UTR. Ribosomes without Rpl13a seem to function normally, bringing up the hypothesis that the ribosome may also serve as storage for regulatory proteins that are released when needed.

Stimulation of cells by TNFα leads to induction of the NF-kB pathway. Rps3 was shown to translocate to the nucleus after TNFα stimulation and interacts there with the NF-kB complex. Phosphorylation of Rps3 by ERK and PKC was also detected, indicating an involvement in different signaling pathways.

1.5.3 Disorders in ribosome biogenesis and diseases

Synthesis of proteins is an essential process in all cells, as is its pre-requisite, the production of ribosome. If these processes are troubled, diseases of the organisms can be expected. More
and more, defective ribosome biogenesis comes into focus for being important in various diseases.

The rate of cell growth and proliferation is proportional to protein synthesis. On one hand, translation can be upregulated to yield more proteins, which is the case in some cancer types. On the other hand, more ribosomes available to the cell simply increase protein synthesis quantitatively. As ribosome biogenesis and its regulation is closely linked to cell growth and proliferation (see section 1.5.2), a link to the abnormal behavior of cancer cells seems evident, especially because the nucleolus, where many steps of ribosome biogenesis happen, is often altered in cancerous cells.

Like for inhibitory mutations in Rb proteins and p53, such changes in regulative pathways lead to increased ribosome biogenesis and to an abnormally high number of ribosomes. As cancer cells usually proliferate much faster than normal cells, they need a larger number of ribosomes to meet the demand of protein synthesis. Generation of rRNA stands at the beginning of ribosome biogenesis, and it is therefore not surprising that rRNA transcription is often upregulated in cancer cells.

Indeed, many cancer cells produce an abnormal number of ribosomes (Montanaro et al. 2008). Decreased activity of the tumor suppressors p53 and Rb proteins lead to an increase in rRNA transcription, and therefore to an increased number of ribosomes (Ruggero and Pandolfi 2003). This happens indirectly by the influence of p53 and Rb proteins on the cell cycle, but also by their direct effects on proteins in the transcription initiation complex. For example, a point mutation in Rb that blocks its interaction with UBF may cause lung carcinomas.

Furthermore, CK2 and the ERK kinase pathway, both hyperactive in certain types of cancer, stimulate rRNA transcription. Consequently, lower levels of the phosphatases that revert the activity of CK2 and ERK have the same effect.

In addition to enhanced rRNA transcription, also the production of ribosomal proteins and trans-acting factors needs to be upregulated. As described above, the transcription factor c-myc regulates synthesis of rRNA, ribosomal proteins and trans-acting factors. In many tumor types, c-myc and other members of the myc family are overexpressed, leading to overproduction of several components of the ribosome biogenesis pathway. Overexpression of NMYC for example leads to elevated levels of nucleophosmin and nucleolin.

A direct involvement of a trans-acting factor in a cancer-linked disease was shown for DKC1. This protein is involved in pseudo-uridine synthesis (part of a H/ACA snoRNA complex), like its yeast homologue Cbf5p, and is also part of the telomerase complex. Mutations in
DKC1 were associated with the disease dyskeratosis congenita (DC), which leads to increased cancer susceptibility, bone marrow failure, and other syndromes. From experiments in mice, it was concluded that the malfunction of DKC1 in pseudo-uridinylation is at least partially responsible for the disease. Translation of a group of mRNAs containing internal ribosomal entry sites (IRES) is impaired in cells missing DKC1, indicating that the pseudo-uridines are necessary for translation start from IRES. The tumor suppressor p27 and other proteins involved in control of cell growth and proliferation are among the affected proteins (Yoon et al. 2006).

A disease that is associated with ribosomal proteins of both subunits is the Diamond-Blackfan Anemia (DBA), characterized by bone marrow failure. A quarter of the patients suffering from DBA have mutations in the Rps19 gene, and also other ribosomal proteins of both subunits were found to be mutated in some cases (Campagnoli et al. 2008). Mutations in these proteins lead to impaired ribosome biogenesis. However, how this then engenders the disease is yet unclear.

A third disease associated with bone marrow failure and ribosome biogenesis, next to DC and DBA is the Shwachman-Diamond syndrome (SDS) (Boocock et al. 2003). In SDS patients, the SBDS protein is mutated. As described in section 1.3.3, the yeast homologue, Sdo1p, is involved in Tif6p release from the pre-60S ribosomal subunit, suggesting that a defect copy of SBDS impairs ribosome biogenesis.

1.6 Perspectives on ribosome biogenesis in yeast and higher eukaryotes

The last ten years have greatly increased the knowledge about ribosome biogenesis in prokaryotes and eukaryotes. Many of the factors involved in this process have been identified. In yeast, purification of pre-ribosomal particles by tandem affinity purifications allowed for analysis of the state of pre-40S and pre-60S factors at different steps in the ribosome biogenesis pathway. This mainly descriptive work necessarily preceded the elucidation of molecular mechanisms, which is now the primary goal in the research in this field.

To achieve this goal, obtaining 3D structures by electron microscopy may be of great support. In two cases described in section 1.3.3, structural investigations (Schafer et al. 2006; Ulbrich et al. 2009) supported the evidence from biochemical experiments for the elucidation of molecular mechanisms in 40S and 60S biogenesis. Localizing trans-acting factors on the surface of pre-ribosomal particles may provide further information which proteins act together in a specific maturation step. Elucidation of molecular mechanism therefore will rely on a combination of biochemical, structural and genetic experiments.
Whereas the study of ribosome biogenesis in yeast has progressed well in the past years, it is still in its infancy in higher eukaryotes. The current assumption is that this very basic process is conserved in throughout the eukaryotes, and hence knowledge about the factors and mechanisms involved in yeast can be transferred to metazoan cells. Initial studies have indeed confirmed that human homologues of yeast trans-acting factors associate to pre-ribosomal particles, and the mammalian trans-acting factors described so far are involved in similar steps of ribosome biogenesis. This however does not tell whether the mechanisms are indeed conserved, and to what extent. Such comparative studies have not been performed up to date. Thereby, investigations into molecular processes in mammalian cells may also provide informations about their development during evolution.

An important pre-requisite for studying molecular details is the knowledge of the proteins involved. The gathering of an inventory of ribosome biogenesis factors in mammals is still ongoing. As the snoRNAs and rRNA precursors are thought to be identified, this concerns mostly proteins. In silico analysis show that for some of the yeast trans-acting factors, multiple homologues in the human genome exist. If all homologues of yeast proteins and additional, previously uncharacterized proteins would participate in ribosome biogenesis, the number of factors would significantly increase compared to yeast, which would present further challenges.

Next to the mere catalog of proteins, an important point will be to study also extra-ribosomal functions of both ribosomal proteins and trans-acting factors. For some mammalian homologues studied so far (Arx1p/Ebp1, GNL3, Rpl23, and others) additional functions outside of ribosome biogenesis have been described. On one hand, these proteins may have simply acquired additional functions during evolution. On the other hand, they may connect ribosome biogenesis to other cellular pathways, as described in section 1.5.

This thesis should contribute to these three aspects of ribosome biogenesis research in mammalian cells – inventory of trans-acting factors, molecular mechanisms and connections between ribosome biogenesis and other pathways. A tandem affinity method in human cell lines is used to isolated human pre-ribosomal particles, whose composition is analyzed by Western blotting and mass spectrometry. This approach is then combined with depletion of specific proteins by shRNA. In the example presented here, the kinase Rio2 was depleted, which leads to accumulation of Rrp12 on pre-40S particles. Furthermore, three pre-60S particles were purified and their composition determined.

In a second part, single proteins are analyzed in more detail. Firstly, the two closely related GTPases Lsg1 and GNL1 as well as Rlp24 are investigated for their role in 60S biogenesis.
Secondly, the role of BCCIPβ in ribosome biogenesis is studied. This protein has been previously described as being involved in double-strand break repair as well as in the p53 and p21 pathways. Starting from the association to Tif6 and Rpl23, its connection to ribosome biogenesis will be investigated.
2. Materials and Methods

2.1 Materials

2.1.1 Protein Names

The table below lists the protein names appearing in the text. For each protein, the name in the NCBI gene database is given as well as the yeast homologue, where existing.

<table>
<thead>
<tr>
<th>Name used in this text</th>
<th>Database name</th>
<th>Yeast homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arx1</td>
<td>PA2G4</td>
<td>Arx1p</td>
</tr>
<tr>
<td>BCCIPα</td>
<td>BCCIP, alpha isoform</td>
<td>Bcp1p</td>
</tr>
<tr>
<td>BCCIPβ</td>
<td>BCCIP, beta isoform</td>
<td>Bcp1p</td>
</tr>
<tr>
<td>CK2β</td>
<td>CSNK2B</td>
<td>Ckb2p</td>
</tr>
<tr>
<td>Dim1</td>
<td>DIMT1L</td>
<td>Dim1p</td>
</tr>
<tr>
<td>Dim2</td>
<td>PNO1</td>
<td>Dim2p</td>
</tr>
<tr>
<td>DUSP12</td>
<td>DUSP12</td>
<td>Yvh1p</td>
</tr>
<tr>
<td>Enp1</td>
<td>BYSL</td>
<td>Enp1p</td>
</tr>
<tr>
<td>GNL1</td>
<td>GNL1</td>
<td></td>
</tr>
<tr>
<td>Ltv1</td>
<td>Ltv1</td>
<td>Ltv1p</td>
</tr>
<tr>
<td>Lsg1</td>
<td>Lsg1</td>
<td>Lsg1p</td>
</tr>
<tr>
<td>Nob1</td>
<td>NOB1</td>
<td>Nob1p</td>
</tr>
<tr>
<td>Noc4</td>
<td>NOC4L</td>
<td>Noc4p</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>NCL</td>
<td>Nsr1p</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>NPM1</td>
<td></td>
</tr>
<tr>
<td>MRTO4</td>
<td>MRTO4</td>
<td>Mrt4p</td>
</tr>
<tr>
<td>Rei1</td>
<td>ZNF622</td>
<td>Rei1p</td>
</tr>
<tr>
<td>Rio1</td>
<td>RIOK1</td>
<td>Rio1p</td>
</tr>
<tr>
<td>Rio2</td>
<td>RIOK2</td>
<td>Rio2p</td>
</tr>
<tr>
<td>Rio3</td>
<td>RIOK3</td>
<td></td>
</tr>
<tr>
<td>Rlp24</td>
<td>RSL24D1</td>
<td>Rlp24p</td>
</tr>
<tr>
<td>Rpl10</td>
<td>RPL10</td>
<td>Rpl10p</td>
</tr>
<tr>
<td>Rpl23</td>
<td>RPL23</td>
<td>Rpl23p</td>
</tr>
<tr>
<td>Rpl23a</td>
<td>RPL23A</td>
<td>Rpl25p</td>
</tr>
</tbody>
</table>
Antibodies against Noc4, Dim1, Dim2, Enp1, Ltv1, Nob1, Rio1, Rio3, Rps2, Rps3, Rps3a, Rrp12, Nmd3-NES, Rpl10, Rpl23a have been described previously (thesis I. Zemp, 2007) or will be described elsewhere. Antibodies against BCCIP, Lsg1, GNL1, Rlp24, Tif6, Sqt1 have been produced at the Institut für Labortierkunde, University of Zurich, using recombinant full length antigen purified from *E. coli*. All antigens where purified under native conditions except for GNL1, which was purified under denaturing conditions. For the BCCIP antibody, full length BCCIPβ was used for immunization. Antibodies against CK2β and MRTO4 where purchased from Santa Cruz Biotechnology (sc-12739, sc-81856), antibodies against the HA tag from Covance (HA-11, MMS-101R). The dilutions used for immunofluorescence are indicated in the table below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-MRTO4</td>
<td>1:300</td>
</tr>
<tr>
<td>α-HA</td>
<td>1:3000</td>
</tr>
<tr>
<td>α-Tif6</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-Sqt1</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-Rlp24</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

Tab. 2.2 Dilutions of primary antibodies in immunofluorescence.

Plasmids for the Protein A/CBP TAP method were prepared by Martina Eicke (M. Eicke, diploma thesis), except for Nmd3-TAP and Lsg1-TAP. Plasmids used for the Strep/HA TAP are denoted e.g. “SH-TAP-Noc4”. Rlp24-YFP was obtained from Thomas Wild.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nmd3-TAP</td>
<td>hNmd3-cTAPup</td>
</tr>
</tbody>
</table>

39
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>5' End Sequence</th>
</tr>
</thead>
</table>
| hNmd3-cTAPdown | hLsg1-cTAPup  
  hLsg1-cTAPdown   | 5'-attaagctttatgggtgcggaggagagc-3'  
  5'-cgccagatcatcattgcagcacttgc-3' |
| Lsg1-TAP    | Noc4_TAPG_fwd  
  Noc4_TAPG_nrev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| SH-TAP-Noc4 | Dim1_TAPG_fwd  
  Dim1_TAPG_nrev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| SH-TAP-Dim1 | Enp1_TAPG_fwd  
  Enp1_TAPG_c_rev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| SH-TAP-Dim2 | MRT04_TAPG_fwd  
  MRT04_TAPG_c_rev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| SH-TAP-Ltv1 | Rei1_TAPG_fwd  
  Rei1_TAPG_nrev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| Arx1-SH-TAP | GNL1_TAPG_fwd  
  GNL1_TAPG_c_rev   | 5'-attaggatccaatgggaggggcggcgg-3'   
  5'-attactcgagctatgactgtagctgag-3' |
| GNL1-SA-SH-TAP | FLJ_EGFP_fwd  
  FLJ_EGFP_rev   | 5'-cattctgatcacaagaggctgactgtagctgag-3'   
  5'-cgatccgctgtactgtgtagctgag-3' |
| Lsg1-GFP    | Lsg1_DN_fwd  
  Lsg1_DN_rev   | 5'-cattctgatcacaagaggctgactgtagctgag-3'   
  5'-cgatccgctgtactgtgtagctgag-3' |
| pTER-NEO-shRps3 | shRps3-a-s: 5'-gatccgcaagatggcagttgcaataattttcatctctgtgagag-3'   
  shRps3-a-as: 5'-agctttcttctttgtgagag-3' |
| pTER-NEO-shRio2 | shRio2-d-s: 5'-gatccgcaagatggcagttgcaataattttcatctctgtgagag-3'   
  shRio2-d-as: 5'-agctttcttctttgtgagag-3' |
Tab. 2.3. Plasmids used in this study. The plasmids GNL1-S51A-SH-TAP and Lsg1-D215N-GFP were generated using the QuikChange protocol (section 2.2.2), generation of the shRNA vectors is described in section 2.2.3.

### 2.1.4 siRNA oligonucleotides

All oligos were used at a concentration of 10 nM (2 pmol oligonucleotide in 2 ml medium).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oligonucleotide name</th>
<th>Sequence</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTER-NEO-shCK2β</td>
<td></td>
<td>5’-gatcccttcacagagatatgtcttgagacatttggaa-3’</td>
<td></td>
</tr>
<tr>
<td>shCKIIb-180-s</td>
<td></td>
<td>5’-agtttcttcacagagatatgtcttgagacatttggaa-3’</td>
<td></td>
</tr>
<tr>
<td>shCKIIb-180-as</td>
<td></td>
<td>5’-agtttcttcacagagatatgtcttgagacatttggaa-3’</td>
<td></td>
</tr>
<tr>
<td>shLsg1-b</td>
<td></td>
<td>5’-gatcccttcacagagatatgtcttgagacatttggaa-3’</td>
<td></td>
</tr>
<tr>
<td>shLsg1-as</td>
<td></td>
<td>5’-agtttcttcacagagatatgtcttgagacatttggaa-3’</td>
<td></td>
</tr>
<tr>
<td>pcDNA5/FRT/TO/nSH-TAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nnTAPG_1</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>nnTAPG_2</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>nnTAPG_3</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>nnTAPG_4</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>pcDNA5/FRT/TO/cSH-TAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ccTAPG_1</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>ccTAPG_2</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>ccTAPG_3</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>ccTAPG_4</td>
<td></td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>Tif6-SH-TAP</td>
<td>Tif6_TAPG_fwd</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tif6_TAPG_c_rev</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>SH-TAP-BCCIPβ</td>
<td>BCCIP_TAPG_fwd_BclI</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCCIPbeta_STOP_rev</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td>SH-TAP-BCCIPα</td>
<td>BCCIP_TAPG_fwd</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bcp-alpha_TAPG_n_rev</td>
<td>5’-attatactcaggaacttttagctgagatggttacctaccagagcg-3’</td>
<td></td>
</tr>
</tbody>
</table>
**Tab. 2.4. Oligonucleotides used for RNA interference.** Rio2-d and Crm1-1 were already described in (Zemp et al. 2009), oligos against both BCCIP isoforms are from (Meng et al. 2007), and the siRNA L21-ME3 is from (Wanzel et al. 2008).

### 2.2 Molecular Cloning

#### 2.2.1 Cloning of plasmids

Coding sequences for the human proteins BCCIPα, BCCIPβ, DUSP12, GNL1, Lsg1, MRTO4, Noc4, Rlp24, Rpl10, Rpl23 and Sqt1 were amplified from a HeLa cDNA library prepared using the Oligotex Direct mRNA Midikit (Qiagen). Coding sequences for the yeast proteins Lsg1p, Nmd3p, Rpl10p, Sqt1p were amplified from whole yeast DNA prepared using standard methods (obtained from Anna Deplazes, Lab of Matthias Peter, IBC). For amplification from cDNA, the Expand High Fidelity PCR system (Roche) was used, and for amplification from plasmid DNA the Vent polymerase (NEB). PCR primers were obtained from Microsynth. All plasmids prepared during this study are listed in tab. 2.3. For restriction digests and ligations, standard enzymes were used (NEB, Fermentas).
2.2.2 Site-directed mutagenesis

To mutate single amino acids in Lsg1 and GNL1, a modified protocol of the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was used. First, PCRs were performed using 100 ng template plasmid, 125 ng of each primer, 0.4 mM dNTP mix and 5 U Pwo polymerase. Reactions were run for 18 cycles (30 sec at 95 °C, 1 min at 55 °C, 9 min at 68 °C). PCR products were digested with 10 U DpnI for 1 h at 37 °C and transformed into heat shock-competent E. coli XL1 blue cells.

2.2.3 Assembling the SH-TAP tag

Vectors pcDNA5/FRT/TO/nSH-TAP and pcDNA5/FRT/TO/cSH-TAP were prepared to allow for N- or C-terminal tagging of the bait protein, respectively. The oligos (tab. 2.3) were assembled using Pwo DNA polymerase, and the resulting fragment was inserted into the KpnI/HindIII sites of pcDNA5/FRT/TO (Invitrogen) to yield pcDNA5/FRT/TO/nSH-TAP or into the ApaI/XhoI sites of pcDNA5/FRT/TO for pcDNA5/FRT/TO/cSH-TAP.

2.2.4 Generation of shRNA vectors

The shRNA vector is based on the pTER vector (van de Wetering et al. 2003). As the original pTER contains a zeocin resistance but the 293T FlpIn TRex cells are still at least partially resistant against zeocin after insertion of a gene in the FRT site (product information, Invitrogen website), the shRNA cloning cassette was transferred to pcDNA3.1+/Neo (Invitrogen) using PvuI/HindIII to generate pTER-Neo. For the insertion of the Rio2-shRNA cassette into the genome, pTER-shRio2 was generated by inserting the DNA duplex from the respective oligonucleotides into the BglII/HindIII sites of pTER-Neo.

2.3 Protein detection

2.3.1 SDS-PAGE and protein staining

For direct or indirect detection, protein were separated according to their molecular mass by denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run in electrophoresis buffer at a constant current of 30-50 mA.

Direct detection of proteins in gels was performed using Coomassie or silver staining. For Coomassie staining, gels were stained with Coomassie staining solution after a brief fixation in 3% acetic acid, followed by extensive washing with dH2O.

For silver staining, gels were incubated in fixation solution for 45 minutes to overnight, followed by glutaraldehyde solution for 45 min to overnight. After washing three times with...
dH2O for 8-10 min, gels were incubated with silver staining solution for 30 min. After rinsing with dH2O, developer solution was added, and the staining reaction was stopped by addition of 50 mM Na₂EDTA.

- **Electrophoresis buffer**: 25 mM Tris base, 133 mM glycine, 1% SDS
- **Coomassie staining solution**: 1% (w/v) Coomassie blue G250, 40% methanol
- **Fixation solution**: 15% (v/v) acetic acid, 30% (v/v) ethanol
- **Glutaraldehyde solution**: 0.5 M NaOAc, 12 mM Na₂S₂O₃, 0.125% glutaraldehyde, 25% ethanol
- **Silver staining solution**: 0.1% AgNO₃, 0.011% formaldehyde
- **Developer solution**: 300 mM Na₂CO₃, 0.011% formaldehyde

### 2.3.2 Western blot analysis

For specific protein detection, samples were transferred to Nitrocellulose membranes (N-2639, Sigma) by semi-dry Western blotting after SDS-PAGE. The transfer was performed for 60-70 min in electrophoresis buffer containing 20% methanol, at a constant current of 1.1 mA times the area of the gel in cm². The membrane was blocked in 5% dry milk in TBT for 45-60 min at RT or overnight at 4°C, and then incubated with first antibodies diluted in 5% dry milk in TBT for 2 h at RT or overnight at 4 °C. After washing three times for 10 min in TBT, horseradish peroxidase (HRP)-conjugated secondary antibody specific for the organism from which the first antibody originated, diluted in 5% dry milk in TBT was added to the membrane for 30 min at RT. Next, the membrane was washed three times with TBT for 5 min each. The chemiluminescence emitted by the secondary antibody in the respective solutions were detected by Super RX films (Fuji).

- **TBT (Tris-buffered Tween)**: 6 mM Tris base, 19 mM Tris HCl, 150 mM NaCl, 0.1% (v/v) Tween® 20
- **Chemiluminescence solution**: 100 mM Tris/HCl pH 8.5, 1.25 mM luminol, 68 µM p-Coumaric acid, 0.01% H₂O₂

### 2.3.3 Mass spectrometry analysis

Single protein bands were excised from Coomassie stained gels and sent in for analysis to Jens Pfannstiel/Iris Klaiber (University Hohenheim, Hohenheim) or to Alexander Wepf.
(IMSB, ETH). For analysis of whole TAP purified complexes, samples were eluted from HA beads with 200 mM Glycin pH 2.2 and neutralized with 100 mM NH$_4$HCO$_3$.

2.4 Protein expression

2.4.1 Expression
All proteins from pET plasmids were expressed in Rosetta E. coli cells (Novagen). Electrocompeotent cells were prepared using standard methods and transformed by electroporation. Pre-cultures were grown overnight at 37 °C in LB medium and diluted the next day in pre-warmed medium. The cultures were diluted 2:1 with cold LB medium (4 °C) when they reached an OD600 of 1.2. The incubator temperature was switched to 25 °C, and after 30 min protein expression was induced for 3-4 hours. Before harvesting, 125 mg PMSF dissolved in Ethanol was added per liter of culture.

For denaturing purification, the temperature was kept at 37 °C throughout the whole procedure.

2.4.2 Native protein purification

E. coli cells were resuspended in lysis buffer (20 ml per liter of E. coli culture), lysed by sonication and the lysate cleared by ultracentrifugation (45 min, 55'000 rpm, Ti70 rotor (Beckman)). Ni-NTA beads (Qiagen) were washed with lysis buffer before and after addition of the cell lysate. Proteins were then eluted with 60% lysis buffer/40% 1M imidazol.

Lysis buffer: 50 mM Tris/HCl pH 7.6, 700 mM NaCl, 2 mM MgCl$_2$, 5% (v/v) glycerol, 20 mM imidazol, 2 mM β-mercaptoethanol.

2.4.3 Denaturing protein purification

E. coli cells were resuspended in buffer A (5 ml per gram E. coli cells) and incubated for 1 hour at RT and then added to Ni-NTA beads (1 ml per gram of E. coli cells) previously equilibrated with buffer A. Proteins were allowed to bind for 2 hours to overnight at RT. Beads were then washed with buffer A and B followed by elution with buffer C.

Buffer A: 6 M GdnHCl, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl pH 8.0
Buffer B: 8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 Tris-HCl pH 6.3
Buffer C: 8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl pH 4.5
2.4.4 Protein rebuffering

Proteins were rebuffered using SpectraPor Dialysis membranes (Spectrum Laboratories) in at least 3 liters of cold buffer. Proteins for antibody production were dialysed to PBS and, if precipitates were formed, treated with a microsonicator to ensure a fine dispersion.

2.5 Preparation of extract from mammalian cells

2.5.1 Preparation of HeLa low salt extracts

Pellets of 5 x 10⁹ HeLa cells (4C Biotech, Belgium) were resuspended in low salt extraction buffer supplemented with protease inhibitors. After cell lysis by dousing, cell debris was pelleted by centrifugation (Sorvall H6000A rotor, 4500 rpm, 15 min, 4 °C). The pellet was extracted once more with the same buffer, and centrifuged again. The supernatants of both extraction steps were combined, supplemented with 250 mM sucrose, shock frozen in liquid nitrogen and stored at -80 °C.

Low salt extraction buffer: 50 mM Tris pH 7.6, 100 mM KCH₂CO₃, 2 mM MgCl₂, 0.15% (w/v) digitonin

2.5.2 Preparation of extracts with NP-40

Cells were washed once with PBS and detached with PBS/0.5 mM EDTA. After harvesting by centrifugation (3 min, 800 g), cells were resuspended in NP-40 lysis buffer and lysed using a douncer (Kimble Kontes, Cat#885300-0015). For volumes below 2 ml, a 20G needle was used. The extract was cleared by centrifugation (12 min, 5000 g).

NP40 lysis buffer: 10 mM Tris pH 7.6, 100 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.5% NP-40 (Fluka, 74385), protease inhibitors (Leupeptin, Aprotinin, Pepstatin), phosphatase inhibitors (Na₃VO₄, NaF)

2.5.3 Hypotonic extracts

Cells were washed once with PBS, then detached with PBS/0.5 mM EDTA and finally washed with 10 mM Tris pH 7.6/10 mM KCl, 2 mM MgCl₂. Lysis of the cells was done in hypotonic lysis buffer by swelling the cells for 20 minutes followed by 5 strokes with a 27G needle. The extract was cleared by centrifugation (12 minutes, 5000 g) and adjusted to 100 mM KCl.
Hypotonic lysis buffer: 10 mM Tris pH 7.6, 10 mM KCl, 2 mM MgCl₂, 1 mM DTT, 0.05% Triton X-100 (Fluka, 93426) protease inhibitors (Leupeptin, Aprotinin, Pepstatin), phosphatase inhibitors (Na₃VO₄, NaF)

2.6 Co-Immunoprecipitations

2.6.1 Coupling of antibodies

From a 9:1 mixture of Protein A:Protein G sepharose beads (GE Healthcare), one ml beads was equilibrated in 50 mM Hepes/KOH pH 8.0, 250 mM NaCl. 2-3 mg antibody (α-hNmd3-NES, α-Lsg1 (polyclonal)) in the same buffer were incubated on the beads for 1 h. Beads were washed twice with 10 ml 50 mM Hepes/KOH pH 8.0, 250 mM NaCl and once with 10 ml 0.15 M Na₂B₄O₇ pH 9. For cross-linking, 10 ml 20 mM dimethyl-pimelimidate (DMP) in 0.15 M Na₂B₄O₇ pH 9 was added to the beads, followed by incubation for 30 minutes. After washing with 0.15 M Na₂B₄O₇ pH 9, beads were incubated with 10 ml 1 M Tris/HCl pH 8.0 to quench the reaction. Then, beads were washed once with 0.2 M ethanolamine pH 8.0, once with 0.15 M Na₂B₄O₇ pH 9 and once with 200 mM glycine/HCl pH 2.2. After rebuffering to PBS, 0.01% thimerosal was added for storage at 4 °C.

2.6.2 Immunoprecipitations with Nmd3 and Lsg1

For testing the interactions between Nmd3 and Lsg1, 500 µl low salt extract was supplemented with 125 mM KOAc and mixed with 500 µl IP buffer and incubated with 40 µl antibody beads suspension for 2 hours at 4 °C. After incubation, the beads were washed three times with IP buffer and eluted with SDS sample buffer. For IPs from ribosome-free extracts, the extracts were centrifuged for 1 hour in a TLA 100.3 rotor (Beckman Coulter) at 70'000 rpm before incubation with the antibody.

IP buffer: 50 mM Hepes/KOH pH 7.5, 250 mM KOAc, 5 mM Mg(OAc)₂

SDS sample buffer: 75 mM Tris pH 8, 20% glycerin, 4% SDS, 50 mM DTT, traces of bromphenol blue.
2.7 Sucrose gradients

2.7.1 Small-scale gradients in TLS55 rotors
Where analysis of UV absorption was not necessary, small gradients in TLS55 rotors (Beckman Coulter) were used. Linear 15-45% sucrose gradients in 10 mM Tris pH 7.6, 100 mM KCl, 2 mM MgCl₂ were prepared in tubes for TLS55 rotors (about 1.8 ml total volume), using a BioComp GradientMaster system (settings: time 1:01; angle 85; speed 20). The gradients were loaded with 100-400 µg extracts (see section 2.5.2), avoiding volumes over 120 µl to ensure proper separation. After centrifugation for 2 hours at 55'000 rpm, fractions of 160 µl were collected and analysed by Western blotting after TCA precipitation.

2.8 Tandem affinity purification (TAP)

2.8.1 TAP using Protein A/Calmodulin binding peptide
Extracts from stable cell lines (see sections 2.5.2) were incubated with 200 µl IgG sepharose, previously washed with TAP buffer 1 for 2 hours at 4 °C. Then, 100 U TEV protease (Gibco) were added for 1 hour at 16 °C in 1 ml TAP buffer 1. The supernatant, filled up to 4 ml with TAP buffer 1 and then supplied with 2 mM CaCl₂, was incubated with 200 µl Calmodulin beads (Stratagene). Beads were washed with TAP buffer 1 supplemented with 2 mM CaCl₂ three times and then eluted with 3x300 µl TAP buffer 1 supplemented with 2 mM EGTA. Combined eluates were precipitated with TCA and analysed by SDS-PAGE followed by silver staining or Western blotting.

TAP buffer 1: 10 mM Tris pH 7.6, 100 mM KCl, 2 mM MgCl₂, protease inhibitors (Leupeptin, Aprotinin, Pepstatin), phosphatase inhibitors (Na₃VO₄, NaF)

2.8.2 SH-TAP using Strep-Tag/HA-Tag
For standard TAP, NP-40 extracts were used (section 2.5.2). For the TAP experiments in combination with shRNA, hypotonic extracts were used prepared according to section 2.5.3. Extracts prepared from stable 293T FlpIn TReX cell lines were incubated for 30 minutes with 25 µl StrepTactin (IBA), per 15 million cells for 30 min, which were washed twice with NP-40 lysis buffer or hypotonic lysis buffer. Beads were washed three times and the elution was performed with 3x300 µl SH-TAP buffer 2 supplied with 2.5 mM desthio-biotin (Sigma, D1411) for 1 minute each. The eluate was directly added to washed HA-agarose (20 µl per 15
million cells) and then incubated for 1 hour. After washing twice with SH-TAP buffer 2 and once with 10 mM Tris pH 7.6, 2 mM MgCl₂, the purified protein complexes were eluted with SDS sample buffer without DTT. Before using the samples for SDS-PAGE, DTT was added to 30 mM final concentration.

SH-TAP buffer 2: 10 mM Tris pH 7.6, 100 mM KCl, 2 mM MgCl₂, protease inhibitors (Leupeptin, Aprotinin, Pepstatin), phosphatase inhibitors (Na₂VO₄, NaF)

2.9 RNA preparation and northern blots

2.9.1 Preparation of total RNA from cells
Total RNA from adherent cells was prepared as described in the RNeasy Mini Kit (Qiagen, 75140) product manual.

2.9.2 Preparation of RNA from SH-TAP eluates
The tandem affinity purification was performed as described under section 2.8.2, except that in addition 40 U RNase inhibitor (Fermentas) was added to all buffers. The first elution was performed with 3x100 µl instead of 3x300 µl. RNA was extracted from the first eluate using the RNeasy Mini Kit (Qiagen, 75140).

2.9.3 RNA agarose gel electrophoresis
RNA gel electrophoresis and Northern blotting were performed by Barbara Widmann. Gel preparation: Agarose was dissolved in RNase free water by boiling in a microwave oven. The solution was mixed by shaking for 2–3 min and boiled again. This was repeated 2-3 times until the agarose was perfectly dissolved (no translucent particles left!). Afterwards the solution was brought to the boil once more and immediately mixed with the respective amount of 50x Tri/Tri buffer. Subsequently, formaldehyde was added and the solution was poured into a gel chamber in a fume hood. The gel was allowed to polymerize for 30 to 45 min.

For separation of RNA on an agarose gel, 1-2 µg RNA was mixed with the same volume of sample buffer and denatured at 65 °C for 10 min. The samples were loaded on a Tri/Tri RNA gel, which was run at 140 V for 5 min and then at a constant voltage of 120 in 1x Tri/Tri running buffer.

50x Tri/Tri buffer: 1.5 M triethanolamine, 1.5 M tricine in H₂O
sample buffer: 1 mM EDTA, 0.04% (w/v) bromphenol blue in 2.1x Tri/Tri buffer
Tri/Tri RNA gel: 1 % agarose, 1.3 % formaldehyde in 1x Tri/Tri buffer

2.9.4 Northern blotting
RNA was transferred from the agarose gel to a nylon membrane (Hybond-N+, GE Healthcare) in a wet blotting apparatus (TE62, Amersham). The transfer was performed at 400 mA in the cold room for 16 h, using 0.5x TBE as transfer buffer. Afterwards, the membrane was allowed to dry for 5 min and RNA was crosslinked to the membrane using a UV crosslinker. For detection of specific rRNA species, the membrane was pre-incubated with hybridization buffer at the probe-specific hybridization temperature (50 °C for 5’ ITS1) for at least 30 min. 1-3 Mcpm of 32P-labeled probe was added to the membrane in fresh hybridization buffer and incubated at the probe-specific hybridization temperature O/N. Next, the hybridization mixture was removed, the membrane was allowed to cool down to RT (in a closed vial to avoid drying-out) and washed twice with buffer 1 at RT for 10 min, followed by incubation with buffer 2 for another 10 min. To detect the signal, the membrane (wrapped in Saran foil) was exposed on a phoshoimager.

Hybridization buffer: 6x SSC, 5x Denhardt’s solution, 0.9 μg/ml tRNA (E. coli) in H2O
50x Denhardt’s solution: 1 % Ficoll 400, 1 % BSA, 1 % PVP-90
buffer 1: 2x SSC, 0.1% SDS
buffer 2: 1x SSC, 0.1% SDS
20x SSC: 3M NaCl, 200mM tri-sodium citrate dihydrate, pH 7.0
1x TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA

2.9.5 Stripping of Northern blots
For de-hybridizing the Northern blot, the membrane was incubated in boiling hot 0.5 % SDS for 5 min and rinsed once with hot 0.5 % SDS. Efficient stripping was verified by exposure of the membrane on a phoshoimager plate.

2.9.6 5’ end labeling of oligonucleotides
10 pmol oligonucleotide were incubated with 10 U T4 polynucleotid kinase (NEB) and 50 μCi γ32P-ATP in the corresponding buffer (total volume 20 μl) for 45 min at 37 °C. Subsequently, the kinase was inactivated by incubation at 65 °C for 20 min. The labeled oligonucleotide was purified using a MicroSpinTM G-50 column (GE Healthcare). The
sequence of the 5’ ITS1 probe used is 5’-CCTCGCCCTCCGGGCTCCGTTAATGATC-3’ (see also Rouquette et al., 2005; note that in this publication an additional T is present after position 24).

2.10 Cell culture

2.10.1 Cell maintenance
Adherent cells were maintained in standard DME medium supplied with 10% FCS and Penicillin/Streptomycin. FlpIn TReX cells for suspension cultures were slowly adapted by increasing the medium from 100 % DMEM to 90% Freestyle medium (Invitrogen, 12338018)/10% DMEM during 6 weeks. Cells in suspension cultures were kept in spin flasks (IBS Integra Biosciences, 182101 for large volumes, 182026 for small volumes).

2.10.2 Transient transfection
HeLa K or FlpIn TReX cells were transfected 24 to 48 hours before usage. For microscopy, cells were grown on cover slips. Per well (960 mm²), 3 µl Fugene 6 (Roche) were mixed with 97 µl DMEM without FCS/Penicillin/Streptomycin. After 5 minutes, the mix was added to 1-2 µg DNA and incubated for further 25 minutes before adding it to the cells kept in DMEM/FCS/Penicillin/Streptomycin.

2.10.3 Transient RNA interference
Transient RNAi was performed on HeLa cells 72 to 48 hours prior to usage. For microscopy, cells were grown on cover slips. Protein depletion was performed with Interferin (Polyplus Transfections, 409-50) using 10 pmol siRNA mixed with 200 µl DMEM -/- and 2 µl Interferin per well (960 mm²). After an incubation of 25 minutes, the mixture was added to the cells kept in DMEM/FCS/Penicillin/Streptomycin.

2.10.4 Stable cell lines using the FlpIn system for tandem affinity purifications
For generation of cell lines stably expressing the TAP-tagged bait protein under control of tetracycline, 0.1 µg plasmid containing the TAP-tagged bait was co-transfected with 0.9 µg pOG44 plasmid (Invitrogen) in HEK 293T FlpIn TReX cells (Invitrogen). Cells were selected for about 2 weeks in standard DME medium containing 100 µg/ml Hygromycin (Invitrogen) and 15 µg/ml Blasticidin (Invitrogen). Afterwards, all colonies were pooled, yielding a polyclonal TAP cell line.
2.10.5 Stable shRNA cell lines
For insertion of the Rio2-shRNA cassette into the genome, the TAP cell lines were transfected with pTER-shRio2 and selected for 2-3 weeks in standard DME medium containing 100 µg/ml Hygromycin (Invitrogen), 15 µg/ml Blasticidin (Invitrogen) and 625 µg/ml G418 (PAA). Single colonies were isolated and tested for knockdown efficiency.

2.10.6 LMB and cis-platin
Inhibition of the exportin Crm1 was performed by adding 20 nM Leptomycin B (LMB, LC Laboratories) to the cells four hours prior to usage. Cis-platin (Sigma) was added in a concentration of 30 mM 17 hours before usage.

2.10.7 Preparation of cells for TAP
Cells in suspension cultures were diluted to a density of about 1.5 million cells per ml at the time of harvesting. Twenty-four hours before harvesting, cells were induced with 0.5 µg/ml tetracycline. Adherent cells were seeded such that they reached confluency when harvesting. For standard SH-TAP, cells were induced with 0.5 µg/ml tetracycline 24 hours before harvesting. When using SH-TAP combined with shRNA, the cells were induced with 1 µg/ml tetracycline 72 hours and with 0.5 µg/ml 40 hours before harvesting, respectively.

2.11 Microscopy

2.11.1 Fixation and permeabilisation of cells for immunofluorescence
For microscopy analysis, cells were grown on glass coverslips. After washing the coverslips once in PBS, cells were fixed with 4% PFA for 15 minutes (α-Enp1, α-Ltv1, α-Noc4, α-Dim1, α-Dim2, α-Rlp24, α-HA), in 100% cold methanol for 5 min (α-Tif6) or in 2%PFA/0.5% Glutaraldehyde for 10 minutes followed by 10 min in 1 mg/ml NaBH₄ (α-Sqt1). After fixation, cells where washed twice with PBS, permeabilized with 0.1%Triton/0.02%SDS in PBS for 5 minutes and again washed twice with PBS. No permeabilisation was necessary for methanol fixations.

2.11.2 Detection
Fixed and permabilised cells were blocked in PBS/2%BSA/10% goat serum for one hour. After incubation with the primary antibody for one hour (see table 2.2 for dilutions), the coverslips were washed three times in PBS. Secondary antibodies (goat α-mouse-Alexa488, Invitrogen, A11001, goat α-rabbit-Alexa488, Invitrogen, A11008) were diluted 1:300 in PBS/2%BSA and applied for 30 minutes. After washing the coverslips three times in PBS,
antibodies were again fixed in 4% PFA, washed once with PBS and mounted on microscope slides using vectashield (Reactolab, H-1000).

2.11.4 Fixation of GFP samples
Cells containing GFP or GFP fusion proteins were washed in PBS and fixed in 4% PFA for 10 minutes. After washing, the coverslips were mounted on microscope slides using vectashield.

2.11.5 Microscopy
For confocal microscopy analysis, pictures were taken at the Light Microscopy Center (ETH Zurich) with a Leica TCS-SP1 laser scanning confocal microscope. Images were processed using Adobe Photoshop (Adobe) or the ImageJ software (http://rsb.info.nih.gov/ij/).
3. Results

The combination of genetic and biochemical methods in yeast has brought a significant advancement in the understanding of ribosome biogenesis in eukaryotes. Thanks to an established tandem affinity purification procedure combined with mass spectrometry, almost 200 yeast trans-acting factors could be identified. However, molecular mechanisms are still unclear for many steps in ribosome biogenesis. In higher eukaryotes, knowledge is even sparser. Currently, it is assumed that both the factors and the mechanisms are conserved throughout evolution and the processes and proteins in human cells are very much like in \textit{S. cerevisiae}. However, it is not clear to what extent this is actually the case.

The goals of this thesis were on one side to identify proteins involved in ribosome biogenesis, and on the other side to investigate molecular mechanisms, both in human cells. A tandem affinity purification procedure is described that allows for efficient isolation of pre-ribosomal particles and determination of their composition by Western blotting and mass spectrometry. In combination with an shRNA approach that allows simultaneous depletion of trans-acting factors, this method presents a basis for further investigations into the role of proteins involved in the assembly of the ribosomal subunits.

For both the small and the large ribosomal subunit, purified pre-ribosomal particles were analyzed by mass spectrometry to gather an inventory of trans-acting factors in human cells. Together with earlier MS analysis of pre-ribosomal particles by immunoprecipitation and an RNAi screen (thesis Thomas Wild), we could show that indeed many yeast proteins involved in ribosome biogenesis have human homologues with presumably similar function, but revealed also a number of previously uncharacterized proteins without homologues in yeast that may also be involved in ribosome biogenesis or regulation thereof.

In addition, three attempts to elucidate molecular mechanisms will be described:

- Rio2 is a kinase involved in later steps of 40S biogenesis. Rio2 has been shown to be involved in the release of pre-40S trans-acting factor from nascent 40S subunits in the cytoplasm. In various experiments, we demonstrated that depletion of this Rio2 leads to an enrichment of the 40S trans-acting factor Rrp12 on cytoplasmic pre-40S particles.

- Lsg1 and GNL1 are members of a GTPase family conserved from prokaryotes to higher eukaryotes. In yeast, all three members of this family are involved in 60S biogenesis. In the mammalian genome, five homologues can be found. Two of them,
Lsg1 and GNL1, are further investigated. For GNL1, an involvement in 60S biogenesis was demonstrated, and a functionally important CK2 phosphorylation site identified.

- BCCIPβ, but not the other BCCIP isoform BCCIPα, was shown to interact with the ribosomal protein Rpl23 and the pre-60S factor Tif6 and influences ribosome biogenesis. BCCIP was previously shown to associate with BRCA2 and p21CIP, to be involved in double strand break repair and in p53 activation.

### 3.1 Tandem affinity purification from human cell lines

Tandem affinity purifications are a very valuable tool for ribosome biogenesis research in yeast. The yield and purity is generally high. For *S. cerevisiae*, a TAP library is available including a large part of the proteome tagged with a C-terminal Protein A/CBP tag. To isolate protein complexes, the cell lysate is first applied to IgG sepharose, followed by elution with TEV protease that cleaves a specific sequence located between the two tags. The eluate is then applied to calmodulin beads, which binds the second part of the tag, the calmodulin binding peptide (CBP). Finally, purified complexes are eluted by the Ca²⁺ complexing EGTA. This library and the easy preparation of large amounts of yeast cell lysate contributed to the success of this method in yeast (Rigaut et al. 1999; Puig et al. 2001), see (Collins and Choudhary 2008) for a review.

For human cells, however, no such established system exists. Because the isolation of considerable amounts of pure pre-ribosomal complexes may help understanding ribosome biogenesis in higher eukaryotes, we set out to establish a TAP system in human cells.

#### 3.1.1 Tandem affinity purifications with the Protein A/CBP tag

In a first attempt to set up a method to purify human pre-ribosomal particles, we adapted the Protein A/CBP usually applied in yeast to human cells. HEK 293 FlpIn TRex cells (Invitrogen) were stably transfected with various *trans*-acting factors fused to the TAP tag. The transcription is induced by addition of tetracycline. This inducible system allows adjustment of the levels of the tagged bait protein to those of the endogenous counterpart. This should reduce the possibility of unspecific interactions due to strong overexpression.

The efficiency of this purification turned out to be rather low: in both steps of the purification, significant losses of the bait protein (fig. 3.1) were observed (see also (Burckstummer et al. 2006) for a comparison of the efficiency of different TAP tags).
Fig. 3.1. A large part of TAP-Enp1 is lost during purification. Samples of both purification steps were analysed by Western blot. “Input IgG” denotes the cleared cell lysate, which was added to the IgG sepharose “FT IgG” the flowthrough after incubation, “bound IgG” what was bound to the IgG sepharose before elution, “beads IgG” the proteins still bound after elution, and “eluate IgG” the eluate from the IgG sepharose. This was also the input for the second purification step. “FT CBP” indicates the flowthrough after incubation with the calmodulin beads, whereas “bound CBP” represents fraction bound to the calmodulin before elution and “eluate CBP” the final eluate. The numbers indicate the percentage of the respective fraction loaded on the gel.

This required large amounts of starting material, which could only be obtained by adapting the cells to suspension culture. The maximal cell densities that were used here were about 2 million cells per milliliter, so from 400 ml of suspension culture about 800 million cells could be harvested. With the 40S trans-acting factors Enp1 and Ltv1 as baits, sufficient protein complex was obtained to detect protein bands on a Coomassie stained gel, which were cut out for MS analysis (fig. 3.2). The proteins detected in the upper part of the gel were all homologues of known yeast trans-acting factors (Rrp12, Tsr1, Nucleolin, Ltv1, Enp1). Except for nucleolins, these components and also the ribosomal proteins in the lower part of the gel were present in approximately stoichiometric amounts compared to the bait proteins. This indicates that a certain number of trans-acting factors are stably bound to the pre-40S particle.
Fig. 3.2. TAP using Ltv1-TAP and Enp1-TAP isolates pre-ribosomal particles. Eluates from tandem affinity purifications with the baits Ltv1 and Enp1 were loaded on SDS-PAGE followed by Coomassie staining. Bands were then cut out and analyzed by MS. The complex from the Enp1-TAP purification was loaded on two lanes due to the large volume of the eluate; bands from both lanes were combined for MS analysis.

To isolate pre-60S and additional pre-40S particles, the trans-acting factors Arx1, Rio2, Lsg1 and Nmd3 were also tested as bait proteins. Purifications with Lsg1-TAP and Nmd3-TAP yielded only the bait protein without interactants (fig. 3.3), when using Rio2 as bait, no proteins at all were purified. With Arx1, a putative pre-60S particle was purified (data not shown).

When working with yeast in TAP experiments, efficiency is not as important, since large amounts of cells can be easily obtained. Another advantage of using TAP in yeast is that a TAP library is readily available. The human cell lines have to be generated first by cloning the genes into the appropriate vector, followed by selection of stable cell lines and finally adaption to liquid cultures, which takes in total 12-15 weeks per cell line.

Because of the mentioned lack of efficiency, this TAP method turned out to be too tedious and too expensive in human cell lines. Thus, experiments with this system were discontinued.
Fig. 3.3. TAP using Lsg1 or Nmd3 isolates only the bait proteins. Eluates from tandem affinity purifications with the baits Lsg1 (A, B) and Nmd3 (C) were loaded on SDS-PAGE followed by silver staining (A) or Western blotting (B, C). Several of the additional bands visible in (A) with Lsg1-TAP are presumably degradation products, as they appear also in a Western blot with a polyclonal Lsg1 antibody (B, see also section 3.3.1 for purification of Lsg1-TAP with the strep/HA method).

3.1.2. Tandem affinity purification with the strep/HA tag (SH-TAP)

Because the protein A/CBP tag turned out to be too inefficient, we looked for a more effective purification method. A central point was that the amount of starting material should be reduced such that usage of adherent cells would be possible (i.e. not more than about 100 million cells). In a study published in 2009 (Glatter et al. 2009; Wepf et al. 2009), Matthias Gstaiger and co-workers presented a combination of two tags (strep tag/HA tag) which allowed for a much more efficient purification of protein complexes. This new TAP tag consists of twice the strep tag (WSHPQFEK) that can bind to avidin, and an HA tag (YPYDVPDYA). Between the tags and between the tag and the protein are flexible linkers to avoid steric hindrance. The HA tag is placed at the very N- or C-terminus for N of the tagged protein, as the interaction between this tag and the HA antibody is thought to be sterically more demanding than the interaction between the strep tag and the interacting avidin molecules.
To purify pre-ribosomal particles with this method, cell lysates from TAP cell lines expressing the tagged bait protein were first incubated with avidin beads. Elution was performed with biotin, and the eluates then incubated with HA antibodies. After a second elution, the purified complexes were ready for analysis (fig. 3.4).

**Fig 3.4. Overview of the SH-TAP procedure.** The cleared cell lysate from induced TAP cell lines is applied to avidin beads. After washing and elution with biotin, the solution is incubated with anti-HA beads for the second purification step. Purified complexes are finally obtained by elution with SDS sample buffer, acidic elution (200 mM glycine pH 2.2) or HA peptide (10 µg/ml).

**Generation of TAP cell lines**

TAP cell lines expressing tagged bait protein were prepared with the HEK 293 FlpIn TRex cell lines mentioned in section 3.1.1. These cells allow a fast generation of stable cell lines expressing the transfected construct under tetracycline control using the Flp recombinase system.

First, vectors based on the pcDNA5/FRT/TO vector (Invitrogen) containing the SH-TAP tag were constructed from oligonucleotides giving pcDNA5/FRT/TO/nSH-TAP and pcDNA5/FRT/TO/cSH-TAP. Then, ORFs of various trans-acting factors were cloned into these vectors, which were subsequently used for the generation of stable cell lines expressing the tagged proteins upon induction with tetracycline. After selection, all foci were pooled to obtain a polyclonal TAP cell line. As the integration of the vectors into the genome should
happen in a directed manner by the Flp recombinase, the genotype of all foci are thought to be identical.

This purification method yielded pure pre-ribosomal particles in sufficient amounts for further analysis (see sections 3.2 and 3.3). It turned out to be important to test the tag on both ends of the bait proteins. Whereas the results were for some proteins independent of the location of the tag (e.g. Ltv1, Nmd3, Lsg1), other proteins showed differences (e.g. Rio1, Enp1, Noc4, GNL1, Sqt1). This included a range of effects from a reduced amount of purified complex to a complete disappearance of the bait protein (data not shown).

**3.1.3 Generation of shRNA cell lines**

In addition to the purification of pre-ribosomal particles with an efficient TAP procedure, we next looked for a way to purify complexes from cells depleted of certain *trans*-acting factors. Changes in particle composition observed after a protein is not available any more in the ribosome biogenesis pathway could give indications about the function of this protein. For example, enrichment of a certain factor on a pre-ribosomal particle purified form cells depleted of a specific enzyme indicates that this enzyme could be necessary for the release of this very factors from the pre-ribosomal particle.

For depletion of specific proteins from human cell lines, transient RNA interference (RNAi) by delivery of sequence-specific RNA oligonucleotides is usually applied. However, for our case this would be too tedious and expensive due to the amounts of cells needed for tandem affinity purification (more than 10 million cells). We therefore decided to introduce an inducible shRNA cassette (van de Wetering et al. 2003) into the genome of the TAP cell lines, which allows for depletion of a protein upon tetracycline induction. Because the expression of the tagged bait protein and the shRNA is under control of the same promoter, interferences between the two systems cannot be avoided. The main issue is that the amount of tetracycline and the time of induction needed for transcription of the shRNA and the tagged bait protein may be different. Whereas the expression of the bait protein was usually induced for 24 hours, RNA interference of up to 72 hours may be needed for efficient depletion of a protein. Induction with tetracycline for three days before purification leads to additional tagged *trans*-acting factor over two to three cell cycles, which could induce changes in the pathway. Additionally, downregulation needs full induction of the system with 1 µg/ml tetracycline. As endogenous *trans*-acting factors are usually present at high levels, induction with this tetracycline concentration does not lead to massive overexpression. However, for proteins expressed at low levels, only about 0.01 µg/ml tetracycline were needed to reach the
levels of endogenous counterpart, which was the e.g. the case for GNL1 (see section 3.4). In such a case, one has to consider the effects of overexpression when purifying particles upon induction of the bait protein with the tetracycline concentrations needed for shRNA-mediated protein depletion.

Preparation of TAP cell lines combined with inducible depletion of Rio2 and Rps3

We decided to apply a combination of TAP with inducible shRNA on two proteins in the 40S biogenesis pathway, namely the kinase Rio2 and the ribosomal protein Rps3. Rio2 was shown earlier to be necessary for cytoplasmic 40S maturation and the release of certain trans-acting factors from nascent 40S subunits (Zemp et al. 2009). Rps3 was shown in yeast to participate in a late remodeling step of pre-40S subunits (Schafer et al. 2006). The goal was to compare purified pre-ribosomal particles from cells with and without depletion of Rio2 or Rps3. This comparison could give indications about structural and functional roles of Rio2 and Rps3.

For the preparation of stable shRNA-Rio2 and shRNA-Rps3 cell lines with the baits Enp1, Ltv1 and Dim2, the pTER/shRNA vectors were transfected into the respective TAP cell lines and the cells subjected to 2-3 weeks of selection. Single clones were then picked and analyzed for depletion of Rio2 and Rps3 (fig. 3.5 and data not shown).

![Fig 3.5. Generation of shTAP clones.](image)

Vectors containing the shRNA cassettes were transfected into FlpIn cells containing various TAP constructs. After two to three weeks of selection, single clones were isolated and the depletion tested after tetracycline addition (3 days, 1 µg/ml). The clones with the strongest depletion were used for further experiments. “l.c.” = loading control.
Taken together, the “traditional” tandem affinity purification method employed in yeast cells turned out to be too inefficient for our purposes. However, adaption of a new combination of tags (strep/HA) was successful. Also the combination of inducible expression of the bait protein with inducible shRNA-mediated protein depletion was achieved. Having these tools at hand, we proceeded to their application to study 40S and 60S biogenesis in mammalian cells.

### 3.2 Tandem affinity purifications with pre-40S trans-acting factors

We first used the tandem affinity purifications to isolate pre-40S particles. On one hand, the goal was to determine the composition of immature ribosomal subunits at different stages. On the other hand, these particles might be used for functional assays. Furthermore, combination of TAP with depletion of specific proteins by inducible shRNA could give indications about their structural and/or functional role.

Based on data from yeast and on previous results using immunoprecipitation of human 40S precursors, the following bait proteins were chosen to isolate pre-40S particles: Noc4, Dim1, Enp1, Dim2, Ltv1 (Milkereit et al. 2003; Schafer et al. 2003; Zemp et al. 2009).

To confirm that the tagged bait proteins were properly incorporated into pre-40S subunits, we compared them with their endogenous counterparts on sucrose gradient centrifugation and immunofluorescence (fig. 3.6). All tested bait proteins migrated like the endogenous proteins on sucrose gradients (fig. 3.6A). However, we noticed that the endogenous proteins were partially displaced to the free protein fraction, indicating that the number of binding sites at pre-40S particles is limited.

The intracellular localization of the endogenous protein and the TAP construct was compared by indirect immunofluorescence using an anti-HA antibody or a polyclonal antibody against the endogenous protein, respectively (fig. 3.6B). For all five cases, no differences were observed. Hence, we concluded that the tag does not influence the localization of the protein, which is a pre-requisite for correct incorporation of the bait proteins into pre-ribosomal particles, and proceeded to the purification of pre-ribosomal particles.
Fig. 3.6. Characterisation of TAP cell lines. Cell lysates were prepared from TAP cell lines induced for 24 hours with 0.5 µg/ml tetracycline as well as untransfected HEK 293 FlpIn TRex cells and fractionated by sucrose gradient centrifugation. The fractions were analyzed by Western blotting using specific polyclonal antibodies (A). To probe the localization of the bait proteins, the endogenous proteins were detected in HeLa cells by indirect immunofluorescence using specific polyclonal antibodies and compared to the localization of
tagged proteins, which were detected using an HA antibody 24 hours after transfection of the TAP constructs (B).

### 3.2.1 Purification of a series of pre-40S particle

To characterize pre-40S particles from human cell lines, we performed tandem affinity purifications using the five bait proteins mentioned in the section before. Silver staining analysis showed that ribosomal particles were successfully co-purified with Dim1, Enp1, Dim2, Ltv1, and to a lower extent with Noc4 (fig. 3.7).

![Fig 3.7. Analysis of pre-40S particles purified using tandem affinity purification from human cell lines. Tandem affinity purifications were performed as described in materials and methods using the baits Noc4, Dim1, Enp1, Dim2 and Ltv1.](image)

In addition to these five proteins, we also tested Nob1 and Rio2, two other proteins previously shown to be involved in 40S biogenesis. Tandem affinity purification of Nob1 only isolated the bait protein without interactants, and no proteins at all were purified using N-terminally tagged Rio2 (data not shown). Interestingly, a GFP-Rio2 construct was functional in
complementing depletion of the endogenous protein (Zemp et al. 2009). This indicates that the unstructured TAP tag could be inaccessible because it is hidden by the three dimensional structure of the protein, or being degraded during the purification procedure.

Purified complexes were analyzed by Western blotting using antibodies against 40S trans-acting factors and ribosomal proteins (fig. 3.8A) as well as by Northern blotting using an ITS-1 probe (fig. 3.8B).

![Western and Northern blot analysis of pre-40S particles](image)

**Fig. 3.8. Western and Northern blot analysis of pre-40S particles.** Samples from fig. 3.6 were loaded on SDS-PAGE and analyzed with various antibodies (A). The relative amounts of the samples were the same as in fig. 3.7. For the Northern blot (B), RNA was isolated from the eluate of the first purification step and analyzed using an ITS-1 probe, which detects all 18S precursors, but not the mature 18S itself. Northern blot performed by Barbara Widmann.
Several conclusions were drawn from the Western blot data:

- Purifications with the bait proteins Enp1 and Dim2 yield similar particles, with Rio1, only associated with the Dim2 particles, as the only difference. For the particle purified with TAP-Ltv1, the levels of Rrp12, Rio3, c21orf70, Dim1 and Noc4 were decreased, which might reflect a more cytoplasmic particle, as at least Rrp12, Dim1 and Noc4 mainly localize in the nucleolus (fig. 3.6, fig. 3.14).

- Ltv1 runs on SDS-PAGE as a double band, of which the upper band is phosphorylated (T. Wild, I. Zemp, unpublished results). Phosphorylation of Ltv1 seems to occur at the pre-ribosomal particle. Whereas only unphosphorylated Ltv1 can be seen in the particle isolated with Noc4, both the phosphorylated and the unphosphorylated band is visible in the other pre-40S particles.

- Dim1 is the only bait protein that also brings down ribosomal proteins of the large subunit. From this experiment, it is not possible to tell whether this reflects an early 90S particle, a whole ribosome (80S particle) or unspecific interactions.

- TAP-Noc4 isolates mainly a dimer with Nop14 (fig. 3.7 and 3.9), which was also found in yeast (Kuhn et al. 2009). Other proteins in this complex like UTP14 or DHX37 are homologous to yeast proteins involved in very early steps of ribosome biogenesis.

Another potential difference of the isolated pre-40S particles is the processing stage of the 18S rRNA precursor. To analyze this, RNA was extracted from the first eluate and analyzed by Northern blotting using an ITS-1 probe (fig. 3.8B). This probe anneals to the spacer at the 3’ end of the mature 18S RNA (fig. 1.6) and therefore detects 18S precursor before final cleavage at site D. The 18SE precursor was detected in all preparations, whereas for TAP-Dim1, and weakly for Enp1-TAP a second band between 26S and 30S was detected. If this indeed represents an earlier intermediate in the 18S rRNA processing pathway, it would mean that TAP-Dim1 and Enp1-TAP purify at least two different populations of particles containing distinct 18S precursors.

Next to Western blotting, the protein complexes isolated by tandem affinity purification with Noc4 and Ltv1 were also analyzed by MS analysis of gel-excised protein bands (fig. 3.9). The Dim2 particle was analyzed both by analysis of single bands and shotgun MS (tab. 3.1). However, shotgun MS of whole protein complexes did not reveal additional, potentially substiochiometric interactants. This is presumably due to undersampling, as most of the detected peptides belonged to ribosomal proteins (data not shown).
Fig. 3.9. MS analysis of two pre-40S particles and initial characterization of c21orf70. Particles were purified as described in the materials and methods section and visualized by Coomassie blue staining. Bands were cut out and analyzed by MALDI-TOF or ESI (A). One of the proteins found, c21orf70, was further analyzed. Depletion of this protein by three different siRNAs lead to partial relocalization of Rps2-YFP from the cytoplasm to the nucleus (B, upper panel) and to a dispersed Enp1 signal (B, lower panel). Both observations indicate an 40S export defect after c21orf70 depletion. The efficiency of the downregulation was controlled by Western blotting (C). In sucrose gradient centrifugation, c21orf70 partially migrates with Rps3 (D), indicating an association to pre-40S particles.
Tab. 3.1. MS results of Dim2 TAP analysis. Shown are MS results from analysis of single bands and an independent shotgun analysis. Ribosomal proteins and common contaminants (keratins) were omitted.

In addition, we also looked in the shotgun MS of the Dim2-TAP for phosphorylated peptides in both ribosomal proteins and trans-acting factors. An overview of these peptides is shown in tab. 3.2.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phosphopeptide</th>
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<tbody>
<tr>
<td>Rrp12</td>
<td>GDSIEEILADpSEDEEDNEEEER</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>pSQPSKTLFVKGL, KVVVSPPpTKK</td>
</tr>
<tr>
<td>Rio2</td>
<td>EGSEFSFpSDGEVAEK</td>
</tr>
<tr>
<td>Nob1</td>
<td>KDDpSDDDGGGWITPSNIK, GEDVPpSEEEEEEENGFEDR</td>
</tr>
<tr>
<td>Rps3</td>
<td>DEILPtpTPISEQK</td>
</tr>
<tr>
<td>Rps6</td>
<td>VRLLLpSKGH, RLpSpSLRApSTSK, VRLLLpSKGH</td>
</tr>
<tr>
<td>Rps11</td>
<td>MpSVHLSPCFR</td>
</tr>
<tr>
<td>Rps27</td>
<td>DLLHPpSPEEEK</td>
</tr>
</tbody>
</table>

Tab. 3.2. Phosphopeptides for certain 40S proteins.

The phosphosites in Rps6 reflect S6 kinase sites, those in Nob1, Rio2 and Rrp12 are in accordance with common CK2 consensus sites, and the threonine in the second peptide from nucleolin is phosphorylated by Cdk1. The other identified phosphosites do not fit to known consensus kinase sites.

C21orf70, a new putative 40S trans-acting factor

In the pre-40S particles purified with the bait proteins Noc4 (fig. 3.9A) and Dim2 (tab. 3.1), a previously uncharacterized protein, c21orf70, was found. The length of this protein is 230 amino acids, giving a calculated molecular weight of 25.3 kDa. The protein is very basic with a pI of 11.1, and therefore putatively an rRNA binding protein. The amino acid sequence is
conserved only among vertebrates, however an iterated protein blast revealed a protein in *S. cerevisiae*, which is similar in length and pI, but shows almost no identity at the level of the primary sequence. Slx9p has been shown to participate in early 40S biogenesis (Bax et al. 2006).

To confirm that c21orf70 is part of the 40S biogenesis pathway, we tested first its association to pre-40S subunits by Western bloting of the particles purified by TAP (fig. 3.8A) and by co-migration with Rps2 in sucrose gradient centrifugation (fig. 3.9C). In addition, we depleted c21orf70 from an Rps2-YFP reporter cell line (Zemp et al. 2009) using three different RNAi oligonucleotides against c21orf70 (fig. 3.9B). In this cell line, Rps2-YFP is expressed upon induction by tetracycline. In steady state, the fluorescent signal is mainly detected in the cytoplasm. Impaired nucleocytoplasmic transport, caused by depletion of factors necessary for export or defects in 40S biogenesis, leads to relocalization of Rps2-YFP to the nucleus. This was the case after depletion of c21orf70, indicating that this protein is involved in early steps of 40S biogenesis or in nuclear export. A shift of the 40S trans-acting factor Enp1 from the nucleolus to the nucleus in HeLa cells depleted of c21orf70, detected by indirect immunofluorescence, confirmed this observation (fig. 3.9B, lower panel).

To summarize this part, we were successful in purifying different pre-40S particles from human cell lines. The isolated complexes were analyzed for their protein and rRNA content. Investigation into one of the mass spectrometry hits, c21orf70, revealed that this uncharacterized protein is a bona fide 40S trans-acting factor, presumably involved in early steps of 40S biogenesis in mammals.

### 3.2.2 Rrp12 is enriched on pre-40S particles after Rio2 depletion

Enzymes like GTPases or kinases play an important role in the biogenesis of ribosomes. Several of these proteins have been described to act in the maturation of both subunits. However, molecular mechanisms describing how these enzymes act are difficult to elucidate. To address this problem, we combined the TAP method described above with inducible knockdown using shRNA cassettes under tetracycline control (see section 3.1.3).

We used this approach for the bait proteins Enp1, Dim2 and Ltv1 with depletion of the kinase Rio2 and the ribosomal protein Rps3 (fig. 3.5). Rio2 is involved in a late step of 40S biogenesis (Rouquette et al. 2005; Zemp et al. 2009), however its substrate and exact function are not known. Rps3 is a ribosomal protein that is thought to join the pre-40S subunit later than most other ribosomal proteins (Ferreira-Cerca et al. 2005; Ferreira-Cerca et al. 2007) (Wild et al. manuscript in preparation).
Fig. 3.10. Analysis of a combination of SH-TAP-Dim2 and shRNA-mediated depletion of Rio2 or Rps3. TAP was performed as described in materials and methods and analyzed by silver staining (A) and Western blotting (B). Cells were seeded four and induced three days before complex purification with 1 µg/ml tetracycline and the eluates analyzed by SDS followed by Western blotting and silver staining.

The depletion of both proteins was fairly efficient, as judged by the Western blot analysis (fig. 3.10B, see input lanes). Comparison of particles isolated with TAP-Dim2 with and without depletion of Rps3 or Rio2 (fig. 3.10) revealed that the particle itself remained intact despite the missing proteins. Besides the strong changes in the levels of Rio2 and Rps3, respectively, the differences were rather subtle. Firstly, the phosphorylation of Ltv1 seemed to be reduced with lower Rio2 and increased with lower Rp3 levels (visible in both silver staining and Western blotting). Secondly, after depletion of Rio2, the amount of Rrp12 on this particle was increased, an observation that was further investigated. Rrp12 is a HEAT-repeat protein whose yeast homologue localizes in the nucleus and the nucleolus and associates to both pre-
40S and pre-60S particles. It binds in vitro to nucleoporins, Ran, and RNA and was therefore proposed to act as an exportin for ribosomal subunits (Oeffinger et al. 2004).

Fig. 3.11. Rrp12 is enriched on pre-40S particles after depletion of Rio2. TAP with Ltv1 as bait was performed with and without depletion of Rio2. Eluates were analyzed by silver staining (A) to confirm the integrity of the particle and by Western blotting (B) to observe enrichment of Rrp12.

To confirm the enrichment of Rrp12 on pre-40S particles after depletion of Rio2, we also used Ltv1 as bait for tandem affinity purification (fig. 3.11), with a similar result. In addition, sucrose gradients were performed from HEK 293 cells with and without depletion of Rio2 (fig. 3.12). In absence of Rio2, Rrp12 was enriched at the 40S peak.
Fig. 3.12. Rrp12 is enriched at the 40S peak in sucrose gradients. Extracts from HEK 293 cells with and without depletion of Rio2 were fractionated by sucrose gradient centrifugation and the fractions analyzed by Western blotting.

In the aforementioned experiments, we observed that Rrp12 was not only enriched on pre-40S particles but could also more efficiently extracted from the cells in absence of Rio2 (Fig. 3.11B, compare Rrp12 in the two input lanes). A reason for this could be that most of Rrp12 is in the nucleolus during steady state (fig. 3.13), and difficult to extract from there. A more efficient extraction would then mean that more Rrp12 is in the nucleus or the cytoplasm and presumably entrapped there on pre-40S particles.

To test this hypothesis, we performed immunofluorescence against Rrp12 in HEK cells (data not shown). However, it turned out to be very difficult to get clear results with this cell line. We therefore analyzed how depletion of Rio2 affects Rrp12 localization in HeLa cells (fig. 3.13).
Fig. 3.13. Rrp12 localizes to the cytoplasm after depletion of Rio2. HeLa cells were transiently transfected with an siRNA against Rio2 or a control siRNA for 48 hours. Proteins were detected by indirect immunofluorescence using polyclonal antibodies. Experiment performed by Ivo Zemp.

Although most Rrp12 still localized to the nucleolus, the cytoplasmic Rrp12 signal in HeLa cells was significantly increased after depletion of Rio2, like it was earlier described for Dim2 and Enp1 (Zemp et al. 2009). We therefore concluded that Rio2 is directly or indirectly involved in the release of Rrp12 from pre-40S particles in the cytoplasm.

To exclude that the observed effect concerning Rrp12 in TAP cell lines was cell line specific, we tried to confirm the enrichment of Rrp12 on pre-40S particles in HeLa cells. For this purpose, we isolated a pre-40S particle by Enp1 immunoprecipitation from HeLa cells with and without depletion of Rio2 (fig. 3.14AB). As for the TAP cell lines, we observed a more efficient extraction of Rrp12 from cells depleted of Rio2, and also enrichment at the pre-40S particle. The cytoplasmic signal of Rrp12 after depletion of Rio2 therefore presumably represents Rrp12 bound to the pre-ribosomal particle and not free Rrp12.

To conclude this chapter, we successfully purified a series of pre-ribosomal particles from human cell lines, and characterized them by Western blotting and mass spectrometry. Based on experiments combining shRNA and TAP, we established the hypothesis that Rio2 may be needed for the removal of Rrp12 from pre-40S particles. This was confirmed by microscopy experiments examining the localization of Rrp12 with and without depletion of Rio2. However, the molecular details of this process and the substrates of Rio2 still remain unclear.
Fig. 3.14. **Rrp12 is enriched on pre-40S particles after depletion of Rio2.** The kinase Rio2 was depleted from HeLa cells by transient RNAi. (A), Western blot analysis of total cells and cell extract. (B), eluates of the co-immunoprecipitation performed with the extracts from (A). (C), sucrose gradient analysis of the same extracts, both analyzed by Western blotting using specific polyclonal antibodies. Experiments performed by Ivo Zemp.
3.3 Tandem affinity purification with 60S trans-acting factors

The comparative analysis of 40S and 60S trans-acting factors by sucrose gradient centrifugation showed that the biogenesis of the small and the large subunit progress independently after cleavage of the early 90S pre-ribosomal particle in the nucleolus. After having purified different pre-40S particles (shown in section 3.2), we attempted similar experiments for the 60S biogenesis pathway.

3.3.1 Overview of the 60S biogenesis pathway

The bait proteins chose to purify pre-60S particles were determined based on homology to known yeast trans-acting factors. However, in contrast to the experiments with pre-40S trans-acting factors, many of the bait proteins tested here did not yield a pre-ribosomal particle in tandem affinity purifications, or only at a very low stoichiometry (Nmd3, Lsg1, GNL1, Tif6, DUSP12, Rlp24, Sqt1). The subcomplexes purified with GNL1 and Tif6 will be described in sections 3.4 and 3.5. Nmd3-TAP and TAP-DUSP12 did not bring down any interactants, whereas Rlp24 could not be isolated at all. Lsg1-TAP co-purified two vesicle associated proteins (VAPA and VAPB), but no ribosomal proteins or trans-acting factors. Interestingly, the drosophila homologue of Lsg1, NS3, was shown to be implicated in neuronal signaling (Kaplan et al. 2008), so Lsg1 might have a secondary role in vesicle trafficking and signaling. As expected, the Sqt1 purification showed a dimeric complex with Rpl10, whose chaperone Sqt1 it is thought to be (Eisinger et al. 1997; West et al. 2005). The interaction between the two proteins was also confirmed in Rpl10/Sqt1 co-expression experiments in E. coli with both the yeast and human proteins (data not shown).

From ten bait proteins tested, only three purifications, using the baits MRTO4, Rei1 and Arx1 yielded amounts of pre-60S particle sufficient for further analysis (fig. 3.15).
Fig. 3.15. **Purifications of pre-60S particles.** Tandem affinity purifications were performed as described in the materials and methods section and analyzed by silver staining (A) and Western blotting (B). Bait proteins are marked with an asterisk in (A).

On the MRTO4 particle, not only Rpl proteins and 60S *trans*-acting factors, but also the 40S *trans*-acting factors Rio2 and Enp1 as well as Rps3 and Rps3a were detected. Although unspecific binding of Rio2, Enp1 or whole subunits was not detected in other experiments, it cannot be excluded that this is the case here. Alternatively, this observation could indicate that this complex represents at least partially a 90S particle. In the final eluates of tandem affinity purifications with the baits Rei1 and Arx1, no Rio2 and Enp1 was found, and no Rps proteins in the Rei1 TAP. However, it cannot be excluded that certain bait proteins are more prone to unspecific binding.

TAP-Rei1 yielded a complex with only Rpl proteins and pre-60S *trans*-acting factors. For Arx1-TAP, Western blot analysis points to an 80S particle, as the levels of *trans*-acting factors are very low and ribosomal proteins of both subunits are present. But also here,
unspecific interactions cannot be excluded, especially because Arx1 might be an RNA binding protein (Squatrito et al. 2006), and therefore may attach unspecifically to mature ribosomes during the purification.

We also analyzed the composition of these particles by mass spectrometry (fig. 3.16). This analysis revealed a considerable number of proteins that are uncharacterized and/or previously not connected with ribosome biogenesis.

**Fig. 3.16. MS analysis of pre-60S particles.** Bands from Arx1, MRTO4 and Rei1 particles were excised and analyzed by MS. Additional proteins detected in the MRTO4 and Rei1 particle are listed in supplemental tab. S1.

### 3.3.2 Lsg1, Nmd3 and Rlp24 in mammalian 60S biogenesis

Lsg1, Nmd3 and Rlp24 have been established as pre-60S *trans*-acting factors in yeast. Nmd3 was shown to be necessary for 60S export both in yeast (Gadal et al. 2001) and human cells
Experiments in yeast showed that the GTPase Lsg1 is necessary for removal of Nmd3 from pre-60S particles in the cytoplasm (Hedges et al. 2005; West et al. 2005). However, the exact mechanisms of the removal and the involvement of other proteins which may act as GEFs or GAPs for Lsg1 remained hidden. Rlp24 shows a very high homology to Rpl24 (hence its name “ribosomal protein like 24”). In yeast, it has been shown to act as a placeholder. Rlp24 loads on pre-60S particles in the nucleolus and is exchanged to Rpl24 in the cytoplasm (Saveanu et al. 2003; Lebreton et al. 2006). We showed that all three proteins are part of pre-60S particles also in human cells (fig. 3.15B) and set out to investigate the molecular roles of these proteins.

To determine how much protein is bound to pre-60S particles, we performed sucrose gradients of cell extract followed by Western blot analysis of the fractions (fig. 3.17). In coherence with the TAP data (section 3.3.1), most of Nmd3 and Lsg1 are not associated to ribosomal subunits and appear therefore in the upper fractions of the gradient. Rlp24, in contrast, is almost exclusively bound to ribosomal particles. The enrichment of Rlp24 in 80S fractions could indicate that Rlp24 also interacts with mature, translating 60S subunits.

As the next step, we looked for an influence of the depletion of Lsg1, Nmd3 and Rlp24 on the cellular localization of proteins involved in 60S biogenesis. On one hand, we used immunofluorescence against the 60S trans-acting factors MRTO4, Tif6 and Rlp24. On the other hand, we used a cell line expressing Rpl35-GFP after induction with tetracycline (Lukas Badertscher, unpublished). If the depletion of a protein leads to changed localization of the reporter, this indicates an involvement of this protein in 60S biogenesis in a way that affects

Fig. 3.17. Nmd3, Lsg1 and Rlp24 associate with 60S particles. Extracts in 100 mM KCl were prepared from HeLa K cells and fractionated on a 15-45% sucrose gradient. Fractions were analyzed by Western blotting using specific polyclonal antibodies.
transport from the nucleolus to the nucleus or from the nucleus to the cytoplasm. Depletion of the exportin Crm1 and the ribosomal protein Rpl23 served as positive controls, as these are expected to strongly influence 60S biogenesis. Crm1 is necessary for the export of both ribosomal subunits from the nucleus to the cytoplasm (Gadal et al. 2001; Thomas and Kutay 2003).

SiRNA-mediated downregulation of Lsg1, Nmd3 and Rlp24 did not change the localization of MRTO4, Tif6 and Rlp24 in a strong manner (fig. 3.18A). In contrast, for reporters of 40S biogenesis, we observe generally stronger delocalizations after depletion of the respective trans-acting factors (fig. 3.14, (Zemp et al. 2009)). Especially the localization of Tif6 hardly changed after the siRNAs applied here.

Interestingly, depletion of Lsg1 led for both MRTO4 and Rlp24 to a slight enrichment of the signal in the cytoplasm (fig. 3.18A). This suggests that Lsg1 is involved in a cytoplasmic 60S maturation step also in human cells, and depletion of this protein may lead to cytoplasmic entrapment of several trans-acting factors. The effect of the depletion of Nmd3 on MRTO4 localization (stronger nuclear signal) is similar to the RNAi against Crm1, however less pronounced. Depletion of Rlp24 finally led only to slight changes in the localization of MRTO4 and Tif6.

RNAi against Lsg1, Nmd3 and Rlp24 affected the localization of the Rpl35-GFP reporter to different extents. Whereas depletion of Lsg1 led to a weak effect on Rpl35-GFP localization, reducing Nmd3, Crm1, L23 and Rlp24 yields a significant accumulation of Rpl35 in the nucleus (fig. 3.18B), indicating that nuclear steps of 60S biogenesis or the nucleocytoplasmic transport depend on the presence of these factors.
Fig. 3.18. Depletion of Lsg1, Nmd3 and Rlp24 affect localization of 60S reporters to different extents. HeLa cells were depleted from Crm1, L23, Nmd3, Lsg1 and Rlp24 using transient RNAi. Seventy-two hours before fixation, cells were transfected with 9 nM siRNA. Immunofluorescence against MRTO4, Rlp24 and Tif6 (A) and Rpl35-GFP (B) were used as reporters for 60S biogenesis.

3.3.3 Lsg1-GFP shuttles between nucleus and cytoplasm
Since depletion of Lsg1 led to a slight accumulation of MRTO4 and Rlp24 and based on the observation in yeast, we assumed that Lsg1 acts in the cytoplasm. However, it could already load to the pre-60S particle in the nucleus and its activity would be triggered after nuclear export. To test this, we transiently expressed Lsg1-GFP in HeLa cells and treated the cells with 20 nM LMB, a small molecule inhibitor of Crm1, for four hours before fixation. If a protein changes the localization from cytoplasmic to nucleoplasmic upon LMB treatment, this reflects that either the protein is trapped in the nucleus bound to a pre-ribosomal particle or is itself exported by Crm1. For Lsg1-GFP, this was indeed the case (fig. 3.19A). This is different to S. cerevisiae, where Lsg1 has been shown to strictly localize in the cytoplasm (Kallstrom et al. 2003), see also (Reynaud et al. 2005). Interestingly, mutating Asp215 in the canonical NKxD GTPase motif to asparagine, which should abolish GTP binding and hydrolysis, had an influence on Lsg1 localization: The Lsg1-D215N-GFP construct localized
to the cytoplasm also after LMB treatment (fig. 3.19A). Proper association of the GFP constructs with pre-60S particles was confirmed by sucrose gradient fractionation of cell lysates (fig. 3.19B).

Fig. 3.19. Lsg1 shuttles between nucleus and cytoplasm. Wildtype and mutants constructs were transfected into HeLa cells 24 hours before fixation (A). LMB in a concentration of 20 nM was applied four hours prior to fixation. The association of the Lsg1-GFP constructs was tested by sucrose gradient fractionation of cell lysates in 100 mM KCl (B).

3.3.4 Lsg1 and Nmd3 interact only in cell lysates containing ribosomal particles

Since *in vitro* binding experiments using recombinant Nmd3 and Lsg1 have failed, we tried to study the interaction between the endogenous proteins in human cells. Co-immunoprecipitations were used to isolate Nmd3 and Lsg1 and the respective interactants from HeLa cell extracts. Both IPs brought down the bait proteins and their respective potential interaction partner, Nmd3 or Lsg1. Rpl proteins were not detected in the eluates (fig.
3.20A), indicating that only a subcomplex of Lsg1, Nmd3 and possibly additional, unidentified proteins was isolated. This is not completely unexpected as most of Nmd3 and Lsg1 stay in the upper fractions in a sucrose gradient and hence are not bound to pre-ribosomal particles (fig. 3.17). However, when the ribosomal subunits were removed from the extract by ultracentrifugation prior to IP, the two proteins do not interact with each other any more. This was also the case when 5 mM EDTA was added to the extract (fig. 3.20B). These two observations may indicate a regulation underlying this interaction, because the simple presence of both proteins is not sufficient for the interaction. However, it is unclear how the presence of ribosomes fosters the interaction between Lsg1 and Nmd3. Other proteins interacting with Nmd3 and/or Lsg1 were not detected in Western blotting and mass spectrometry (data not shown).

**Fig. 3.20. Nmd3 and Lsg1 interact directly or indirectly with each other.** Nmd3 and Lsg1 antibodies covalently coupled to beads were incubated with low salt extracts and the eluates analyzed by Western blotting. A, comparison between co-immunoprecipitations from total extract and from the supernatant of ultracentrifuged extract. B, comparison of co-immunoprecipitations from extract with and without addition of 5 mM EDTA.

### 3.3.5 Effects of Lsg1 depletion on pre-60S particles

Lsg1 was shown to be necessary for the release of the export adaptor Nmd3 from 60S subunits in the cytoplasm in yeast (Hedges et al. 2005; West et al. 2005). To investigate the role of Lsg1 in human cell lines, we compared the levels of Nmd3 on a pre-ribosomal particle

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**Table:**

<table>
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<tr>
<th></th>
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<tr>
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**Fig. 3.20:** Nmd3 and Lsg1 interact directly or indirectly with each other. Nmd3 and Lsg1 antibodies covalently coupled to beads were incubated with low salt extracts and the eluates analyzed by Western blotting. A, comparison between co-immunoprecipitations from total extract and from the supernatant of ultracentrifuged extract. B, comparison of co-immunoprecipitations from extract with and without addition of 5 mM EDTA.
isolated with an Rlp24 immunoprecipitation from untreated and Lsg1 depleted cells (fig. 3.21). In this co-immunoprecipitation, ribosomal proteins of both subunits were detected as well as 60S trans-acting factors. It can therefore be concluded that this IP isolates a pre-60S particle. Whether the detected Rps proteins represent a 90S particle, a translating ribosome (80S) or unspecific binding of mature 40S subunits cannot be concluded. Nmd3 was indeed enriched in the eluates of the co-immunoprecipitations from cells where Lsg1 had been downregulated by transient RNAi compared to untreated cells and cells depleted of the Lsg1 homologue GNL1. This indicates that Lsg1 is necessary for the release of Nmd3 from pre-60S particles.

Fig. 3.21. Nmd3 is enriched on a pre-ribosomal particle isolated by Rlp24-IP. HeLa cells were depleted of Lsg1 or GNL1 by using specific siRNAs transfected 72 hours before preparation of lysates. As a control, untreated cells were used. The lysates were incubated with Rlp24 antibodies covalently coupled to protein A/G beads. Eluates were analyzed by Western blotting. Experiment performed by Michaela Gerber.

3.3.6 Rlp24 interacts with Importin-β/Importin 7

Rlp24 has been described in yeast to be a placeholder for the ribosomal protein Rpl24. The exchange of Rlp24 and Rpl24 occurs in the cytoplasm, whereas Rlp24 loads to pre-60S particles in the nucleolus. We used Rlp24 co-immunoprecipitations as a tool to isolate pre-60S particles (see section 3.3.5). Pulldown experiments using a recombinant zz-Rlp24 fusion protein and HeLa cell extract (fig. 3.22A) revealed ribosomal proteins of both subunits in the eluate (data not shown). Like for the Rlp24 immunoprecipitation shown in the section before, this presence of ribosomal proteins could reflect unspecific binding to mature ribosomal subunits or an association of Rlp24 to 80S particles (see section 3.3.2). Interestingly, this experiment showed that Rlp24 interacts with Importin β and Importin 7 in a RanQ69L•GTP sensitive manner (fig. 3.22A, lower panel). The dimeric complex of these two importins has been shown to import very basic proteins into the nucleus, like ribosomal proteins or histones.
(Bauerle et al. 2002; Harel and Forbes 2004). Rlp24, with a pI of 9.7, fits also in this category.

To confirm the need of Importin β/Importin 7 for nuclear import of Rlp24, we tested whether simultaneous depletion of these two proteins would lead to a reduced import of Rlp24 in cells. Both importins were downregulated by RNAi for 64 hours. Twelve hours before fixation, cells were transfected with Rlp24-YFP (T. Wild). Consistent with the hypothesis that the two importins contribute to the nuclear import of Rlp24, the ratio of nuclear to cytoplasmic signal was reduced in the cells treated with siRNA oligonucleotides against Importin β/Importin 7 (fig. 3.22B). However, a significant fraction of Rlp24-YFP was still imported into the nucleus, which may be due to inefficient depletion of the importins or to an import pathway independent of Importin β and Importin 7.

Fig. 3.22. Importin β and Importin 7 are candidate importins for Rlp24. KH extracts was incubated with recombinant zzRlp24 and IgG sepharose. Eluates were analyzed by SDS-
PAGE followed by silver staining and Western blotting (A). HeLa cells depleted of Importin β/Importin 7 were transfected with Rlp24-YFP to visualize reduction of Rlp24 import (B).

To summarize this chapter, we were successful in purifying pre-60S particles from human cell lines with three different bait proteins. In two of the three preparations 40S ribosomal proteins were detected, which raises concerns about unspecific interactions (see also discussion, section 4.3). Analysis of the purified particles by Western blotting confirmed that homologues of yeast pre-60S trans-acting factors are present on the pre-ribosomal particles. By an extensive MS analysis, several previously uncharacterized proteins were found, or proteins for which so far no involvement in ribosome biogenesis has been shown. However, for these proteins additional experiments have to be performed (similar to those presented for c21orf70 in section 3.2.1) to confirm a contribution to mammalian 60S biogenesis.

In a second part, three different pre-60S factors, Rlp24, Nmd3 and Lsg1 were investigated in more details. However, despite some interesting findings, no definitive conclusions about the functions of these proteins could be drawn.

### 3.4 GNL1 associates with and is phosphorylated by casein kinase 2

Enzymes like kinases or GTPases play important roles in the biogenesis of both the small and the large subunit. On family of GTPases involved in 60S biogenesis is distinguished by a circular permutation of the GTPase motifs in the amino acid sequence (Reynaud et al. 2005). In *S. cerevisiae*, the family comprises three members, Lsg1p, Nug1p, Nog2p, which are all implicated in ribosome biogenesis (Bassler et al. 2001; Kallstrom et al. 2003; Saveanu et al. 2003). In higher eukaryotes, the number of family members is increased. In *D. Melanogaster*, there are four of these GTPases (NS1 to NS4), in the human genome five members can be found (Lsg1, GNL1, GNL2, GNL3, GNL3L). The putative evolutionary relationship is shown in tab. 3.3.

<table>
<thead>
<tr>
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<td>Lsg1</td>
<td>NS3</td>
<td>Lsg1</td>
</tr>
<tr>
<td></td>
<td>NS4</td>
<td>GNL1</td>
</tr>
<tr>
<td>Nog2</td>
<td>NS2</td>
<td>GNL2</td>
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<tr>
<td>Nug1</td>
<td>NS1</td>
<td>GNL3</td>
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<tr>
<td></td>
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<td>GNL3L</td>
</tr>
</tbody>
</table>

Tab. 3.3. Relationship between the circularly permutated GTPases in yeast, drosophila and humans.
Whereas the involvement of the three yeast GTPases in ribosome biogenesis has been shown previously, this is not the case for the homologues in higher eukaryotes. GNL3 (nucleostemin) has been shown to be implicated in rRNA processing in human cells (Romanova et al. 2009), as well as its homologue in *D. melanogaster*, NS1 (Rosby et al. 2009; Tsai and Meng 2009).

Because Lsg1 and GNL1 are closely related, and as both proteins associate with pre-ribosomal particles (fig. 3.15B, fig. 3.21, supplementary tab. S1), we next addressed the question whether GNL1 would also be involved in 60S biogenesis.

Microscopic analysis of 60S reporters (Rpl35-GFP, immunofluorescence with antibodies against MRTO4, Rlp24, Tif6) did not show any delocalizations after depletion of GNL1 by RNAi (data not shown), indicating that the depletion was not sufficient to cause an effect, or that GNL1 acts in redundant roles with other proteins, or that GNL1 function is simply not necessary for nuclear 60S maturation or pre-60S export. However, GNL1-D228N acts as a dominant-negative variant of GNL1, as overexpression of this construct in the Rpl35-GFP reporter cell line caused an export defect. As the D228N point mutation should abolish GTPase activity of GNL1, this suggests that the GTPase activity of GNL1 is needed for proper 60S biogenesis.

![Figure 3.23](image.jpg)

*Fig. 3.23. Overexpression of GNL1-D228N causes a pre-60S export defect.* Rpl35-GFP reporter cell lines where transfected with GNL1-wt-TAP and GNL1-D228N-TAP constructs
24 hours prior to fixation. The expression of the TAP construct was detected by indirect immunofluorescence using an HA antibody.

To further characterize GNL1, we looked whether it migrates at the 60S peak in sucrose gradient centrifugation. However, only minute amounts of GNL1 could be found in the 60S fractions, indicating that most of GNL1 is not associated with pre-60S particles (fig. 3.26, upper panel). The depletion of GNL1 by shRNA, in combination with tandem affinity purifications with the baits MRTO4 and Rei1, showed no effect on the composition of pre-ribosomal particles (data not shown).

### 3.4.1 GNL1 associates with the casein kinase 2 holoenzyme

Initial experiments showed that only very little GNL1 associated to pre-60S particles in sucrose gradient centrifugation and in TAP experiments. We investigated therefore whether other protein complexes would be attached to GNL1, using immunoprecipitation of GNL1 as well as tandem affinity purification. Whereas co-IPs did only yield very little GNL1 and no detectable interactants (data not shown), TAP with a C-terminally but not N-terminally tagged version of GNL1 associated with the casein kinase 2 holoenzyme (fig. 3.24).

![Fig. 3.24. GNL1 associates with the CK2 holoenzyme.](image)

Tandem affinity purifications were performed as described in the materials and methods section, single bands excised from a Coomassie-stained SDS-PAGE gel and analyzed by MS. For GNL1, one phosphorylation was detected at serine 51, which is the only CK2 consensus site in the GNL1 sequence.
Because of the association of GNL1 with CK2, we looked whether phosphorylated peptides could be found in the MS data. Indeed, the only CK2 consensus site (Olsten and Litchfield 2004) in GNL1 at serine 51 was phosphorylated (fig. 3.24, right).

In the TAP cell lines, GNL1-TAP is highly overexpressed compared to the endogenous protein at the standard tetracycline concentrations used for induction (0.2-0.5 µg/ml medium). Others trans-acting factors examined showed an expression of the tagged protein comparable to the endogenous protein at this tetracycline concentration. For GNL1-TAP, the tetracycline concentration required to reach the levels of endogenous protein was only 0.005-0.01 µg/ml (data not shown). This was also the case for GNL1 point mutants (see section 3.5.2).

However, the CK2 association is not due to abnormally high GNL1 levels. The same band pattern was also seen when the TAP was performed with the bait proteins induced at 0.005 µg/ml tetracycline (data not shown). So, either the GNL1-TAP fusion protein is unusually stable and highly expressing, or the levels of endogenous GNL1 are lower than those of other trans-acting factors. Interestingly, the GNL1 mRNA bears a 5’-UTR of about 1000 nucleotides, whereas most trans-acting factors have UTRs below 100 nucleotides. This long UTR could contain sequences that negatively regulate GNL1 translation.

3.4.2 Mutation of serine 51 to alanine leads to an increased association of GNL1 with a pre-60S particle

The phosphorylation of GNL1 at serine 51 could be important for the association of the protein to the pre-60S particle. As the parts of the ribosomal subunit not covered by ribosomal proteins are negatively charged due to the rRNA backbone, phosphorylation of GNL1 could lead to a weakened interaction by electrostatic repulsion. To test this hypothesis, we mutated the Ser51 to alanine to prevent phosphorylation. This mutation indeed led to an increase of 60S proteins attached to GNL1-TAP, both trans-acting factors and ribosomal proteins, as shown by silver staining and Western blotting (fig. 3.25). Still, the stoichiometry of GNL1-TAP to the proteins of the pre-60S particles was very high (fig. 3.25A), indicating either a weak binding of the phospho-deficient mutant to the pre-60S particle or a limited number of particles to which GNL1 can bind. It is so far unclear whether GNL1 binds the pre-60S particle and the CK2 holoenzyme simultaneously.
**Fig. 3.25. Mutations of serine 51 to alanine leads to slightly increased association of 60S proteins.** Stable TAP cell lines were generated with GNL1-wildtype and a GNL1-S51A point mutation, both with the TAP tag at the C-terminus. The tandem affinity purifications were performed as described in the materials and methods section, and the isolated particles analyzed by silver staining (A) and Western blotting (B), using specific polyclonal antibodies.

To confirm that phosphorylation of GNL1 by CK2 leads to a reduced binding of GNL1 to pre-60S particles, sucrose gradients in presence and absence of active CK2 were performed. Depletion of the kinase should lead to reduced phosphorylation of GNL1 and therefore an enrichment of the protein on the 60S fractions. For this experiment, CK2β, the structural subunit of the CK2 tetramer, was depleted from HeLa cells by transient RNA interference. Cell lysates were fractionated by sucrose gradient centrifugation and the fractions were analyzed by Western blotting to show that GNL1 indeed enriches at the 60S peak (fig. 3.26). However, this was also the case for other proteins such as Lsg1. This could indicate either a general mechanism, in which trans-acting factors are removed from nascent ribosomal subunits by CK2 phosphorylation, or that removal of GNL1 from pre-60S particles is a prerequisite for further processing steps and dissociation of other 60S trans-acting factors.
Fig. 3.26. GNL1 and other trans-acting factors are enriched on the 60S peak after depletion CK2β depletion. CK2β was depleted from HeLa cells by transfecting a specific siRNA 72 hours before harvesting the cells. Extracts from si-CK2β and control treated cells separated by sucrose gradient centrifugation and the fractions analyzed by Western blotting.

For GNL1, a role in 60S biogenesis was at first, based on preliminary experiments, not obvious. Only the overexpression of a GTPase dead form led to an export defect in the Rpl35-GFP reporter cell line. Because GNL1 localizes to the cytoplasm (fig. 3.23) and does not shuttle in and out of the nucleus in a Crm1-dependent manner (data not shown), the effect on 60S biogenesis effect is presumably indirect, e.g. by the release and subsequent recycling of a factor necessary for pre-60S export.

Tandem affinity purification with GNL1 showed that this GTPase associates with and is phosphorylated by CK2. The phosphorylation seems to regulate the binding of GNL1 to pre-60S particles. By this phosphorylation, CK2 may trigger a series of processing events in the cytoplasm that eventually leads to mature 60S subunits. Another possibility is that CK2 phosphorylation is a general mechanism for removing trans-acting factors from the pre-60S subunit. However, further investigations are required before any conclusions can be drawn.
3.5 BCCIPβ forms a trimer with Tif6 and Rpl23 and may be involved in 60S biogenesis

Whereas the primary goal of the tandem affinity purifications was to isolate whole pre-ribosomal particles, we encountered also subcomplexes of only a few proteins. These subcomplexes may give indications about the role of the involved proteins, like for the purification of GNL1-TAP that indicated an involvement of CK2 in ribosome biogenesis (section 3.4). Also another of the 60S trans-acting factors that were used to purify pre-60S particle, Tif6, isolated a complex of only few protein. The isolated trimer will be described and initially characterized in this part.

3.5.1 Tif6 is part of a subcomplex together with L23 and BCCIPβ

Purification of Tif6-TAP revealed a pre-60S particle and a trimeric complex consisting of the β isoform of BCCIP (BRCA2 and Cdc1 interacting protein) and the ribosomal protein L23 (fig. 3.27). BCCIP exists in two isoforms, BCCIPα and BCCIPβ. The first 263 amino acids are the same, whereas the C-terminus (about 60 amino acids) is different.

BCCIPα has been shown to interact with the tumor suppressor protein BRCA2 and p21 (Ono et al. 2000; Liu et al. 2001). This protein, also known as CDKN1A, Cip1 or Waf1, is an inhibitor of the CDK1 and CDK2 kinases (Abbas and Dutta 2009). BRCA2 is involved in genome stability, especially double-strand break repair together with the recombinase RAD51 (Thorslund and West 2007). In addition, BCCIP also may have direct influence on the transcription factor activity of p53 (Meng et al. 2007).

L23, on the other hand, is not only a ribosomal protein, but was shown to inhibit the p53 ubiquitin ligase HDML2 (Zhang and Lu 2009). Also the ribosomal proteins L5 and L11 seem to exert this function. Currently, it is thought that by this mechanism, interrupted biogenesis of ribosomes is relayed via increased levels of free ribosomal proteins to the p53 pathway, which then blocks proliferation of the cell. Human Tif6 and BCCIP were shown to interact in a large-scale MS assay (Ewing et al. 2007), BCCIP and L23 in a large-scale yeast two-hybrid assay (see http://interactome.dfci.harvard.edu), whereas the homologues of Tif6 and L23 in S. solfataricus interact as well (Benelli et al. 2009).

The trimeric complex of BCCIPβ/Tif6/L23 was confirmed by tandem affinity purification with TAP-BCCIP. Only the β, but not the α isoform of BCCIP yielded the trimeric complex (fig 3.27). Whereas Tif6-TAP also associates with a pre-60S particle, this is not the case for BCCIPb (fig. 3.27B). Performing TAP with BCCIPβ constructs where 10, 20 or 40 amino
acids from the C-terminus were eliminated indicate that the binding occurs at the very C-terminus of BCCIPβ (fig. 3.27C).

Dimeric interactions between L23 and BCCIP as well as between L23 and Tif6 were confirmed in co-expression experiments in *E. coli* (data not shown).

**Fig 3.27: Tif6, BCCIPβ and Rpl23 form a trimeric complex.** Tandem affinity purifications were performed with Tif6-TAP, TAP-BCCIPα and TAP-BCCIPβ and analyzed by silver staining (A) and Western blotting (B). Tif6-TAP purifies substoichiometrically pre-60S particle, with stoichiometric bands of BCCIPβ and Rpl23, which were confirmed by MS. BCCIPβ but not BCCIPα yields the same trimeric complex. Truncation constructs of BCCIPβ reveal that the very C-terminus of BCCIPβ might be the part interacting with Rpl23 and Tif6 (C, experiment performed by Daniel Alper).

### 3.5.2 Depletion and overexpression of BCCIPβ influences 60S reporters

Like for other 60S *trans*-acting factors, the influence of BCCIPβ depletion on 60S biogenesis was probed with immunofluorescence against 60S *trans*-acting factors and a Rpl35-GFP reporter cell line. For some of the reporters tested here, slight changes in their localization were seen after depletion of BCCIPβ, but not for depletion of BCCIPα, indicating that only
the β isoform has indeed an effect on ribosome biogenesis. Whereas the MRTO4 signal is only affected by depletion of Crm1, depletion of BCCIPβ reduces the nucleoplasmic signal for both Tif6 and Rlp24 (fig. 3.28). For Rlp24, the effect may be due to a general influence on 60S biogenesis. For Tif6, the slight reduction of the nucleoplasmic signal could be due to the absence of the interaction partner BCCIPβ, which also localizes to the nucleoplasm (fig. 3.29).

![Image](image_url)

**Fig. 3.28: Depletion of BCCIPβ slightly affects localization of Rlp24 and Tif6.** Proteins were depleted from HeLa cells by transient RNAi. Cells were fixed after 72 hours and trans-acting factors detected by indirect immunofluorescence. To deplete either BCCIPα or BCCIPβ, two previously described siRNA oligos were used (Meng et al. 2004) (note that the siRNA oligo against BCCIPβ in this publication differs from the BCCIPβ sequence in the NCBI database, hence a slightly different siRNA oligo was used here, see materials and methods).

Next, we also tested whether depletion of BCCIPβ affects localization of Rpl35-GFP in the reporter used above. Depletion of neither isoform of BCCIP changed the localization of Rpl35-GFP (fig. 3.29A). However, overexpression of a TAP-BCCIPβ construct (the same used for the tandem affinity purifications described in section 3.5.1) led to an increased nuclear GFP signal in the reporter cell line (fig. 3.29B). This could indicate that high levels of
BCCIPβ block export of pre-60S particles, either directly or indirectly by sequestering Rpl23 and/or Tif6.

**Fig. 3.29:** Overexpression of BCCIPβ but not BCCIPα leads to a 60S export defect. Both isoforms were depleted from the Rpl35-GFP reporter cell line using transient RNAi and the GFP signal recorded after 72 hours (A). Rpl35-GFP cell lines were transfected with TAP-BCCIP constructs and fixed 24 hours later. The transiently transfected constructs were detected by indirect immunofluorescence using an HA antibody (B).
4. Discussion

4.1 Tandem affinity purification of human pre-ribosomal particles

Ribosomes exert a crucial role in every human cell, and hence ribosome biogenesis is a very fundamental process. However, published studies about this subject are sparse, and ribosome assembly in humans is still poorly understood. In yeast and in prokaryotes, recent years brought significant progress in understanding the actors and some of the mechanisms of ribosome biogenesis. To what extent this knowledge can be transferred to higher eukaryotes is unclear. Mammalian cells are more complex, contain about four times more genes, and hence more intricate interaction maps and cellular pathways. Although ribosome biogenesis is a fundamental process, it can be assumed that also ribosome biogenesis is considerably more complex in mammals compared to yeast, involving additional factors and regulative layers. Thus, gathering an inventory of factors participating in ribosome biogenesis and assigning their function to different steps in the synthesis of the large and small ribosomal subunit in human cells is of great interest. This thesis should contribute to this undertaking by presenting an approach to systematically purify pre-ribosomal particles of both subunits at different stages of maturation, and by characterizing some novel trans-acting factors in ribosome biogenesis.

In a first step, we set out to establish an efficient method to purify pre-ribosomal particles from human cells. On one hand, this allowed to identify the factors involved in human ribosome biogenesis and to compare them with the data obtained in yeast. On the other hand, purified pre-ribosomal particles can be a starting point for functional studies on ribosomal particle maturation.

The TAP tag used in yeast, consisting of a Protein A domain and a CBP tag, was not efficient enough to purify pre-ribosomal particles from human cell lines with reasonable efforts. Hence, we adapted the strep/HA tag combination, originally introduced by Matthias Gstaiger and co-workers (Glatter et al. 2009; Wepf et al. 2009), which turned out to be much more efficient for the purification of protein complexes. Using the FlpIn system, TAP cell lines were generated that express the tagged bait protein under control of tetracycline. Inducible expression allows for the adjustment of the expression levels of the tagged bait protein to those of its endogenous counterpart, which may be important to reduce unspecific interactions. In this system, also mutated bait proteins that may exhibit functional differences
can be used, like for example seen with the GNL1 phosphodeficient S51A mutant (see section 4.4)

Identification and initial characterization of novel 40S trans-acting factors

Five different pre-40S particles and three pre-60S particles were purified using TAP and their composition analyzed by Western blotting and mass spectrometry (fig. 3.7, fig. 3.8; see section 4.3 for discussion of the pre-60S particles). For such analysis, the higher purity of protein complexes isolated with tandem affinity purifications is an advantage in comparison to complex isolation by immunoprecipitations using antibodies to specific trans-acting factors.

Besides factors already previously associated to ribosome biogenesis, we identified putatively new trans-acting factors like the uncharacterized protein c21orf70. For this protein, we could show that it not only associates with pre-40S particles purified by TAP (fig. 3.8), but also co-migrates with the 40S peak in sucrose gradients (fig. 3.9). Further, depletion of c21orf70 leads to export defects of pre-40S particles (fig. 3.9).

The role of other proteins like G3BP1 or GNB2L1 in 40S biogenesis still has to be investigated. G3BP1 regulates the levels of certain mRNAs and was recently shown to stabilize p53 (Kim et al. 2007). GNB2L1/RACK1 was previously shown to associate with 40S particles and was proposed to be a ribosomal protein (Gerbasi et al. 2004). GNB2La has also been implied in signaling pathways (Coyle et al. 2009). So, GNB2L1 and G3BP1 perhaps link 40S biogenesis to other pathways.

Particle composition and staging

The pre-40S particles purified with the baits Enp1 and Dim2 were highly alike in protein composition and pre-rRNA content. They showed similar levels of the pre-40S factors Rrp12, Rio2, Rio3, Ltv1, Enp1, c21orf70, Nob1, Noc4 and Dim2. Interestingly, Rio1 was found exclusively at the Dim2 particle. This may be due to a direct interaction of Dim2 with Rio1, or indicates a small subpopulation among the particles isolated with TAP-Dim2, to which Rio1 binds. The similar particle composition is in agreement with the comparable localization of Enp1 and Dim2 in HeLa cells (fig. 3.6). The only difference in localization, the small fraction of Dim2 detected to the cytoplasm, may also be the cause for the different Rio1 association with the purified particles.

Compared to the Enp1-TAP and the TAP-Dim2 pre-40S particles, those isolated with Ltv1 as bait protein showed a weaker enrichment of Rrp12, c21orf70, Noc4, Dim1 and Rio3. Among
others, these proteins might make up the difference between a cytoplasmic particle, which TAP-Ltv1 is thought to isolate due to its cytoplasmic localization, and the presumably nuclear/nucleolar particles isolated with the baits Dim2 and Enp1. However, it is difficult to define from which cellular compartment the purified particles originate. In steady-state, Enp1 and Dim2, for example, localize predominantly to the nucleolus and to the nucleus, and consequently the purified particles should represent a mixture of nuclear and nucleolar particles. Yet, this contrasts with the observation that only 18SE rRNA was found in the particles, and no earlier 18S precursors like the 21S pre-rRNA or longer precursors. Additional studies focusing on the extraction of various rRNA species and proteins will be required to clarify this point.

With respect to TAP-Dim1 and TAP-Noc4, it is interesting that both localize to the nucleolus like Enp1 and Dim2, but yield completely different particles. The analysis of the Noc4 particle was difficult due to weak extraction of the bait protein. MS analysis revealed that the particle most likely represents an early pre-40S particle, as components of the SSU processome were found therein. The complex purified by TAP-Dim1 was the only one that contained proteins of the large ribosomal subunit (fig. 3.7). The presence of Rpl proteins suggest that either a 90S pre-ribosomal particle was purified, or that the particle contains mature 60S subunits, which might indicate association of Dim1 with translating 80S ribosomes. It can also not be excluded that the presence of Rpl proteins is a result of unspecific interactions.

The differences between the Dim1 and Noc4 particle on one side and the Enp1-TAP and TAP-Dim2 particle on the other side, despite the common predominantly nucleolar localization of all four factors, might be explained by the localization of these trans-acting factors to different parts of nucleolus. It is conceivable that Dim1 and Noc4 localize to the fibrillar centre (FC) or the dense fibrillar component (DFC), whereas Enp1 and Dim2 locate to the granular component (GC). This might also explain differences in the extraction efficiency.

**Outlook**

The tandem affinity purification method of pre-ribosomal particle presented here turned out to be successful for our purposes. The generation of cell lines and the purification itself were fast and efficient, and usually took not more than four to five weeks for cloning and selection. The novel putative trans-acting factors detected here will need additional characterization to
confirm their potential role in 40S biogenesis. Using them as bait proteins in further TAP experiments will help to complete the inventory of human 40S biogenesis factors.

4.2 Combining TAP with protein depletion by shRNA

Tandem affinity purification yields the composition of a specific pre-ribosomal particle, but by itself no mechanistic data. To approach this, we decided to combine tandem affinity purification with depletion of specific proteins using inducible shRNAs. Particles were therefore purified out of cells lacking a certain enzyme or a structural protein. Analysis of changes in particle composition after the depletion of a specific factor might contribute to the elucidation of its function.

Depletion of Rio2 impairs release of Rrp12 from cytoplasmic pre-40S particles

Rrp12, which was shown in yeast to be involved in nuclear export of both subunits (Oeffinger et al. 2004), was enriched on TAP-Ltv1 particles after depletion of the kinase Rio2 (fig. 3.11). We hypothesized that Rrp12 is trapped on a cytoplasmic pre-40S particle, which was confirmed by immunofluorescence experiments (fig. 3.13). Rrp12 therefore also seems to be released from nascent 40S subunits in a Rio2-dependent maturation step in the cytoplasm. Likewise, the 40S trans-acting factors Enp1, Nob1, Ltv1 and Dim2 accumulated in the cytoplasm after depletion of Rio2 (fig. 3.13 and (Zemp et al. 2009).

Depletion of Rps3 does not affect pre-40S particles

Rps3 binds to the “head” of the 40S subunit, close to the D site in the 18-SE pre-rRNA. At this site, the last, cytoplasmic cleavage of the pre-18S rRNA occurs. Depletion of Rps3 caused Enp1 to accumulate in the cytoplasm (T. Wild et al., manuscript in preparation). This indicates that Rps3 is needed for a cytoplasmic step in pre-40S maturation. The combination of TAP with shRNA-mediated depletion of Rps3 revealed that Rps3 is not required for the integrity of particles purified with TAP-Ltv1 or TAP-Dim2 (fig. 3.10 and data not shown). In both the silver gel and Western blot analysis, the band pattern and the protein levels remained about constant, while Rps3 was clearly absent. This suggests that Rps3 might only be required for the overall structure of mature 40S particles and for cytoplasmic maturation, i.e. release of trans-acting factors and 18SE to 18S rRNA processing, but not for earlier pre-40S assembly.
**Outlook**

Taken together, tandem affinity purifications coupled to depletion of proteins by shRNA allow for a systematic study of ribosome biogenesis in human cell lines. Whereas generation of cell lines is fairly quick and convenient, subsequent analysis of the particles requires specific antibodies or quantitative mass spectrometry methods, like SILAC (Matthiesen and Carvalho 2010), which allow for unbiased measurements of changes in particle composition. Notably, a number of protein kinases are involved in ribosome biogenesis. The identification of phosphopeptides in protein complexes may thus be a valuable tool for the identification of kinase substrates. In various pre-ribosomal particles purified by tandem affinity purification, we tried to determine phosphorylation sites by mass spectrometry, either from gel-excised protein bands or by shotgun MS. For Rio2, Rrp12, Nob, Nucleolin, Rps3, Rps6, Rps11 and Rps27, phosphorylated residues could be identified, interestingly several of them at CK2 consensus sites, implying a role for this kinase in ribosome biogenesis. However, further analysis is required to prove their functional relevance. This was successful for the Ser51 phosphorylation by CK2 in the GTPase GNL1 (see section 4.4), for which a functional role could be determined.

### 4.3 Investigations into 60S biogenesis

Like for the pre-40S particles, different bait proteins were tested for purification of pre-60S particles. Notably, only three bait proteins, namely Arx1, MRTO4 and Rei1 purified 60S precursors. The other examined proteins, Lsg1, Nmd3, Rlp24, Sqt1 and DUSP12 yielded only the bait protein, subcomplexes or nothing at all. Apparently, it seems more difficult to find suitable bait proteins for pre-60S than for pre-40S purification. Sucrose gradient analysis (fig. 3.17, compare with fig. 3.6) showed that 60S trans-acting factors generally have larger pools of protein not associated with pre-ribosomal particles than 40S trans-acting factors, which likely explains the difficulties to purify pre-60S particles.

**Characterization of human pre-60S particles**

MRTO4-TAP co-purified a particle containing several 60S trans-acting factors, namely Lsg1, Nmd3, Arx1, Tif6, Rlp24, but also the 40S proteins Rio2, Enp1, Rps3 and Rps3a (fig. 3.15). Whereas Lsg1, Nmd3, Arx1 and Tif6 also associated with the TAP-Rei1 particle, Rlp24 did not and also no Rps proteins. In addition, this particle contained traces of GNL1, which is presumably a cytoplasmic protein (fig. 3.23). Thus, we assume that the MRTO4 particle is an
The early pre-60S particle, as Rlp24 and MRTO4 localizes mainly to the nucleolus (fig. 3.18), whereas TAP-Rei1 represents a cytoplasmic pre-60S particle.

The efficient purification of MRTO4-TAP indicates that this predominantly nucleolar protein can be easily extracted from the cells, like the nucleolar proteins Dim2 and Enp1, and unlike Noc4. Further investigations may reveal which proteins localize to which nucleolar substructure and at which state the pre-ribosomal particles can be found in the different nucleolar compartments.

When using Arx1-TAP as bait protein, ribosomal proteins of both subunits were purified, and the levels of trans-acting factors were significantly lower than for the TAP-Rei1 and MRTO4-TAP particle. Because Arx1 is mostly found in the free protein fractions on sucrose gradients, it cannot be excluded that this represents unspecific binding of mature subunits.

MS analysis of the pre-60S particles components revealed several proteins without homologues in yeast, as well as uncharacterized proteins and proteins with functions not related to ribosome biogenesis. For example, ILF2 and ILF3, which were found at the MRTO4 and Rei1 particles, have been shown to form a heterodimer, to bind to interleukin-2 promoters and to be part of the pre-miRNA processing Drosha complex (Sakamoto et al. 2009). Whether they act in both miRNA and rRNA processing or whether they bind unspecifically to pre-ribosomal particles via their RNA binding domains remains to be shown. Interestingly, the eukaryotic elongation factor 2 (EEF2) was found in all three isolated pre-60S particles. The GTPase EEF2 acts in the translocation of aminoacyl-tRNAs from the ribosomal A-site to the P-site during translation. Thus, if the interaction is specific, then the tested bait proteins either interact also with translating ribosomes or EEF2 already binds to pre-60S particles.

To continue investigation into 60S biogenesis, we will one hand look for additional bait proteins that might be used for the isolation of pre-60S particles. On the other hand, their rRNA content should be analyzed to determine the maturation state of the 28S rRNA, but also to look for 18S rRNA and its pre-cursor, which might clarify why 40S proteins can be found in these purifications.

The quest for specific binders

An important issue for the isolation of protein complexes is to avoid unspecific binding of contaminants during the purification procedure. For the method presented here, purifications were performed in 100 mM potassium chloride, a salt concentration that is presumably not high enough to prevent unspecific binding. While background binding to the beads can...
presumably be excluded (fig. 3.7 and fig. 3.8, “no bait” lane), it is still possible that proteins or mature subunits bind to the particles that are physiologically attached to the bait protein. Pre-ribosomal particles contain exposed charged surfaces (negative charge of the phosphate backbone of the rRNA and positive charges of the ribosomal proteins), to which proteins or mature subunits can bind during or after cell lysis. Unspecific binding of mature 40S subunits to the purified complexes during the isolation of pre-60S particles and vice versa could be avoided by fractionating the cell lysates in sucrose gradients before TAP. To exclude unwanted binding of proteins, the salt concentration can be raised in the buffers used for TAP. However, higher salt concentrations may cause the dissociation of physiological components only weakly interacting with pre-ribosomal particles. This trade-off has to be considered when choosing conditions for purification of protein complexes from cell lysates.

4.4 GNL1 and Lsg1, two GTPases in 60S biogenesis

The putative GTPases GNL1 and Lsg1 belong to a family of GTPases characterized by the circular permutations of the canonical GTPase motifs in the primary sequence. This protein family has five members in the human genome and four in D. melanogaster. In yeast, all three members of the family, Nog2, Nug1 and Lsg1, are involved in 60S biogenesis (tab. 3.3). Lsg1p was demonstrated in yeast to be necessary for the release of Nmd3 from pre-60S particles in the cytoplasm (Hedges et al. 2005). In this thesis, we tried to elucidate the molecular details of the functions performed by the closest human homologues of Lsg1p, GNL1 (33% identity with Lsg1p) and Lsg1 (40% identity with Lsg1p).

Human wildtype Lsg1, but not the GTPase dead mutant, was shown to shuttle in and out of the nucleus (fig. 3.19), unlike its yeast counterpart. As only about one third of all Lsg1 in the cell is bound to pre-60S particles (fig. 3.19B), it is not possible to tell from this experiment whether Lsg1 is exported from the nucleus as part of a pre-60S particle or whether this finding indicates a mechanism to keep Lsg1 out of the nucleus, where its presence might be harmful. Further insights why the GTPase dead mutant behaves differently could not be found.

Depletion of Lsg1 led to an enrichment of Nmd3 on pre-60S particles (fig. 3.21), and Lsg1 interacted with Nmd3 in co-immunoprecipitations of the endogenous proteins (fig. 3.20). This indicates a link between Lsg1 and Nmd3 in human cells. Interestingly, presence of pre-ribosomal subunits in the cell lysate seems to be required for the interaction, although it does not necessarily need to be mediated by the pre-ribosomal particle. This could for example mean that Lsg1 and Nmd3 can only interact in a conformation that is induced by binding to
the ribosomal subunit. Further experiments testing the influence of guanosine nucleotides or phosphatases on the interaction were not conclusive (data not shown).

To conclude, we could not derive a definitive model on Lsg1 function from the data obtained here. Further investigations may focus on the interaction between Nmd3 and Lsg1, and how this interaction is regulated. This might clarify whether Nmd3 and Lsg1 are imported to the nucleus as a dimer. Then, it would be interesting to determine the trigger of Lsg1 activity in the cytoplasm, which eventually leads to GTP hydrolysis and Nmd3 release.

**GNL1, a 60S trans-acting factor phosphorylated by CK2**

The second-closest homologue of Lsg1p in human cells, GNL1, was only weakly detected on TAP-purified pre-60S particles (fig. 3.15), and does not migrate with the 60S peak in sucrose gradient centrifugation (fig. 3.26, upper panel). As also the depletion of GNL1 affected the localization of 60S trans-acting factors and Rpl35-GFP in HeLa cells only slightly, an involvement of this protein in 60S biogenesis seemed not obvious. However, the overexpression of GNL1-D228N, carrying a mutation in the NKxD GTPase motif that should abolish GTPase activity, resulted in relocalization of Rpl35-GFP from the cytoplasm to the nucleus (fig. 3.23). This indicates that the GTPase activity of GNL1 is important for proper nuclear maturation or export of nascent 60S subunits.

To investigate whether GNL1 interacts with a subset of ribosomal proteins or trans-acting factors, we performed tandem affinity purifications with a GNL1-TAP bait. In this experiment, GNL1 was associated with the CK2 holoenzyme, composed of one α, one α’ and two β subunits (fig. 3.24). CK2 is a ubiquitous protein kinase and has been shown to phosphorylate about 300 different targets. Generally, it acts in favor of cell cycle progression and growth. A positive effect on ribosome biogenesis would therefore not be surprising. Whereas CK2 has not been implied previously to ribosome biogenesis itself, it promotes rRNA transcription by phosphorylating various proteins involved in rRNA transcription like UBF, TIF-1A or members of the SL1 complex.

MS analysis revealed that the only CK2 consensus site in GNL1, S/T-D/E-x-D/E (Olsten and Litchfield 2004), at Serine 51 was indeed phosphorylated. To investigate the role of this phosphorylation, we performed TAP with a phosphodeficient GNL1-S51A mutant. In contrast to wildtype GNL1, this form bound to a bona fide pre-60S particle (fig. 3.25), indicating that phosphorylation of GNL1 by CK2 leads to its release from the nascent 60S subunits.
To further investigate whether phosphorylation of GNL1 by CK2 releases GNL1 from nascent 60S subunits, we analyzed the distribution of 60S trans-acting factors on sucrose gradients with and without depletion of CK2. In lysates from cells in which CK2β, the structural subunit of CK2 was downregulated, several trans-acting factors showed increased association to pre-60S particles (fig. 3.26). On one hand, this result confirms that binding of GNL1 to pre-60S particles is regulated by CK2. On the other hand, it indicates that CK2 phosphorylation could be a general way to release trans-acting factors from nascent 60S subunits, or that release of GNL1 triggers further processing steps that lead to dissociation of other 60S trans-acting factors. Consequently, CK2 would act in later stages of ribosome biogenesis to promote formation of mature ribosomes (fig. 4.1).

Fig. 4.1: Model of the role of CK2 in late 60S biogenesis. Phosphorylation of GNL1 by CK2 is necessary for the release of GNL1 and subsequently other pre-60S factors.

To what extent CK2 is involved in other steps of ribosome biogenesis is so far unclear. As shown in tab. 3.2, also in other proteins (Nob1, Rrp12, Rio2) phosphorylated serines or threonines at CK2 consensus sites were found. The kinase could therefore promote ribosome biogenesis at different steps.

Taken together, the experiments in this work indicate that GNL1 and its GTPase function are linked to ribosome biogenesis. This is best explained by postulating that GNL1 helps the release of a factor necessary for nuclear export, similar to the function of Lsg1. Furthermore, dissociation of GNL1 from the pre-60S particle relies not only (or not at all) on its GTPase activity, but on phosphorylation by CK2, indicating a role for CK2 in 60S maturation.

Further experiments will focus on the effect GNL1 has on other 60S trans-acting factors, for example by looking for trans-acting factors that are enriched in the cytoplasm after GNL1-D228N overexpression. Additionally, the role of the CK2 phosphorylation at Ser51 should be
further characterized. It will be interesting to dissect how this phosphorylation influences other cytoplasmic 60S processing steps like the release of Nmd3.

4.5 BCCIPβ, a protein with diverse functions

Tif6 has been shown to participate in 60S biogenesis in yeast, where it is phosphorylated by the CKI homologue Hrr25p (Ray et al. 2008). In addition, it blocks translation initiation by not allowing the association of the 40S and 60S subunit (Miluzio et al. 2009). TAP with Tif6 as bait yielded a pre-60S particle, but also a trimeric complex together with the β isoform of BCCIP and Rpl23 (fig. 3.27). This trimer was confirmed by tandem affinity purification of BCCIPβ.

The protein BCCIP exists in two isoforms, alpha and beta, which might have different functions. The α isoform interacts with the tumor suppressor proteins BRCA2 and p21 (Ono et al. 2000; Liu et al. 2001). Like BRCA2, BCCIP is also involved in DNA double-strand break repair. Furthermore, BCCIP may act on p53 activity (Meng et al. 2007). However, the existing studies not always address whether the respective functions are performed by both isoforms or not. The yeast homologue of BCCIP, Bcp1p, has been shown to be required for the export of Mss4, a lipid kinase (Audhya and Emr 2003). Recently, Bcp1p was shown to be involved in pre-28S rRNA processing (Li et al. 2009).

In our experiments, the α isoform of BCCIP, which differs in the last 60 amino acids at the C-terminus from the β isoform, did not show any interaction with Tif6. Further experiments suggested that the very C-terminus of BCCIPβ is necessary for binding of Rpl23 and Tif6. We also tried to reconstruct the trimeric complex from recombinant proteins expressed in E. coli. Whereas direct interactions between Rpl23 and Tif6 and between Rpl23 and BCCIPβ could be shown by co-purification, the interaction Tif6-BCCIPβ and also the trimer were not reproducible. This might indicate that this interaction is regulated by post-translational modifications of Tif6 and/or BCCIPβ that are not available in E. coli.

Because BCCIPβ interacts with a ribosomal protein and a trans-acting factor of the large subunit, we investigated whether it has any effect on 60S biogenesis. Depletion of both isoforms did not affect localization of MRTO4 (fig. 3.28) and Rpl35-GFP (fig. 3.29). Immunofluorescence against Rlp24 and Tif6 showed a slightly weaker nuclear signal after depletion of BCCIPβ, but not BCCIPα (fig. 3.28). This could mean that the interaction with BCCIPβ is necessary for proper localization of Tif6, while the effect on Rlp24 localization is more likely indirect, e.g. through an early 60S processing defect, as no direct connection between Rlp24 and BCCIPβ was found.
Interestingly, overexpression of BCCIPβ in the Rpl35-GFP reporter cell line caused enrichment of the Rpl35-GFP in the nucleus, indicating a dominant negative effect of BCCIPβ on pre-60S assembly or export. Again, this effect was specifically obtained for the β, but not the α isoform. This might be explained by the finding that BCCIPβ forms a complex with Rlp23 and Tif6, whereas BCCIPα does not.

To conclude, the data collected here and in the cited articles suggest that BCCIPβ has a conserved role in 60S biogenesis, whereas BCCIPα is connected to p21 and BRCA2 function. However, as Rpl23 is a regulator of p53, BCCIPβ may also work in pathways regulating growth and proliferation by acting on Rpl23.

Two studies have previously shown that Tif6 binds to the large subunit via Rpl23 (Benelli et al. 2009; Gartmann et al. 2010), which is confirmed by the data presented here. BCCIPβ may thus chaperone Tif6 and/or Rpl23 and facilitate assembly of these proteins to the nascent 60S subunits, or, in contrary, block 60S maturation in the nucleus. This function might be connected to the anti-proliferative role of BCCIP in regard to p53 activation.

Further research on BCCIP should analyze its role in ribosome biogenesis, which seems to be exerted by BCCIPβ. On the other hand, the influence on the p53 and p21 pathways and on double-strand break repair should be investigated.
## 5. Supplemental material

| M1 | EEF2 (3618), NSUN2 (456), Nucleolin (365), MARS (111), SRPK1 (102), DDX50 (81), HNRPNPUL1 (80), RRP1 (77) |
| M2 | ILF3 (2226), XRCC6 (2206), KIAA0776 (1487), TNPO1 (983), IFIH1 (919), nucleolin (844), TBL3 (838), EEF2 (674), C1orf25 (660), QARS (610), SDAD1 (555), DHX15 (500) |
| M3 | XRCC6 (2332), RECQL (651), EIF2AK2 (511), GNl3L (427), PES1 (340) |
| M4 | LRRC47 (1519), FARS (1421), RECQL (682), DDX5 (596), MRTO4 (494), GNl3L (431), SYNCRIPl (430) |
| M5 | EIF3L (1443), TRIM26 (1359), GNl3L (1347), EIF3D (797), IGF2BP1 (749), G3BP1 (627), CDK5RAP3 (495), STAU1 (490), EXOSC9 (415), TRIM25 (357), EIF2A (357), FARS (324), EEF2 (268) |
| M6 | SERBP1 (2289), FARS (1451), Rei1 (1037), STAU (852), C22orf28 (591), PRPF19 (401) |
| M7 | EIF2S3 (1302), DDX47 (1239), RPL4 (1039), SERBP1 (732), nucleolin (565), RUVBL2 (450), RNMTL1 (323) |
| M8 | RPL4 (2724), YBX1 (1181), EIF3E (1046), EIF2B2 (859), Nob1 (603) |
| M9 | Arx1 (3954), Rpl3 (3471), Rpl4 (744), Rpl3L (413), PAK1IP1 (231), Enp1 (215), Lsg1 (158), EIF3G (141) |
| M10 | ILF2 (2075), Rpl4 (937), Arx1 (903), SERBP1 (665), p37 AUF1 (549), DDRGK1 (494), PHF6 (490), RPL3 (424), EIF3G (333), MRTO4 (317), CSNK1E (303), RPSA (279) |
| M11 | RPSA (5364), MRTO4 (1273), DRG1 (832), RPL4 (429), EIF3M (400), EBNA1BP2 (317) |
| M12 | MRTO4 (1277), RPL6 (470), SEC61A1 (364), RFC3 (358), RPSA (335), NPM1 (278) |
| M13 | MRTO4 (2387), RPSA (1599), NPM1 (985), RFC4 (757), EIF2S1 (754), RPLP0 (256), Ltv1 (243), Rps2 (220), EIF2B2 (204), SEC61A1 (167) |

| R1 | EEF2 (3623), NSUN2 (1203), Nucleolin (424), MARS (302), SRPK1 (183) |
| R2 | KIAA0776 (2553), ILF3 (1576), XRCC6 (1389), QARS (768), SDAD1 (550), IFIH1 (479), STRBP (340), DNAJC21 (334), C1orf25 (308), Lsg1 (262), TBL3 (261) |
| R3 | ABCF2 (1558), DNAJC21 (959), DDX3X (604), GNl1 (253) |
| R4 | XRCC6 (2159), ZNF622 (1094), GNl3L (873), KARS (697), RECQL (549), DDX17 (450), EIF2AK2 (416), EXOSC9 (323), FARS (254), RARS (214) |
| R5: Rei1 (3324), FARS (3123), RECQL (1099), EIF2AK2 (950), DDX5 (840), RARS (655), DDX55 (620), EXOSC9 (460), RPN1 (284), SYNCRIPT (225), E2IG3 (164) |
| R6: Rei1 (2992), CDK5RAP3 (1117), EXOSC9 (593), TRIM25 (518), IGF2BP1 (508), GNL3L (436), STAU1 (422), DDX55 (372), EIF3L (315), G3BP1 (239), FARS (219), EIF3D (201) |
| R7: SERBP1 (2797), FARS (1510), STAU1 (795), c22orf28 (313), PRPF19 (297) |
| R8: ILF2 (1700), DDRGK1 (821), Arx1 (315), PHF6 (236), HNRNPD (232), RPL4 (232), RPL6 (162), RPL3 (123) |
| R9: RPSA (1729), RPL6 (274), DRG1 (177) |
| R10: NPM1 (877), RPL6 (817), EIF2S1 (513), RFC4 (317), DUSP12 (304), RPLP0 (169) |

Tab S1. **MS results from MRTO4 and Rei1 TAP.** Numbers denote Mowse scores from the MS analysis.
6. Abbreviations

ATP  Adenosine triphosphate
BSA  Bovine serum albumine
DBA  Diamond-Blackfan anemia
DC   Dyskeratosis congenita
DMEM Dulbecco’s modified Eagle medium
DMSO Dimethyl sulfoxide
DNA  Desoxyribonucleic acid
DTT  Dithiothreitol
EDTA Ethylene diamine tetraacetate
FCS  Fetal calf serum
GAP  GTPase activating protein
GEF  Guanosine exchange factors
GTP  Guanosine triphosphate
GTPase GTP hydrolyzing protein
IF   Immunofluorescence
IP   Immunoprecipitation
IRES Internal ribosome entry site
ITS  Internal transcribed spacer
HEPES 2-(4-(2-Hydroxyethyl)-1-piperaziny1)-ethansulfonic acid
kDa  Kilodalton
LB   Luria broth
LMB  Leptomycin B
LSU  Large subunit
mRNA Messenger RNA
NAD  Nicotinamide adenine dinucleotide
NES  Nuclear export signal
NOR  Nucleolus organizing region
NPC  Nuclear pore complex
O/N  Overnight
PAGE Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
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<th>Abbreviation</th>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Pol I</td>
<td>RNA polymerase I</td>
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<td>RNA polymerase I</td>
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<tr>
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<td>RNA polymerase I</td>
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<tr>
<td>ppGpp</td>
<td>guanosine 3’, 5’-bis(diphosphate)</td>
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<td>Polyvinylpyrrolidone 90</td>
</tr>
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<td>Ribosomal DNA</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>rRNA</td>
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<td>RNA interference</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Ribosomal protein of the small subunits</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>Room temperature</td>
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<td>Sodium dodecylsulfate</td>
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<td>Small hairpin RNA</td>
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7. References


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