Linking nuclear import of ribosomal proteins with ribosome assembly: The role of the “escortin” Tsr2 during ribosome biogenesis

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presented by

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2015
To my family
Wir glauben, Erfahrungen zu machen, aber die Erfahrungen machen uns.

_Eugène Ionesco_
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SUMMARY
From Schütz et al., 2014.

Within a single generation time a growing yeast cell imports ~14 million ribosomal proteins (r-proteins) into the nucleus for ribosome production. After import, it is unclear how these intrinsically unstable and aggregation-prone proteins are targeted to the ribosome assembly site in the nucleolus. Here, we report the discovery of a conserved nuclear carrier Tsr2 that coordinates transfer of the r-protein eS26 to the earliest assembling pre-ribosome, the 90S. In vitro studies revealed that Tsr2 efficiently dissociates importin:eS26 complexes via an atypical RanGTP-independent mechanism that terminates the import process. Subsequently, Tsr2 binds the released eS26, shields it from proteolysis, and ensures its safe delivery to the 90S pre-ribosome. We anticipate similar carriers – termed here escortins – to securely connect the nuclear import machinery with pathways that deposit r-proteins onto developing pre-ribosomal particles.
ZUSAMMENFASSUNG

Aus Schütz et al., 2014.

STATEMENT OF CONTRIBUTION

The work presented in this PhD thesis results from a collaborative effort. For the part “A RanGTP-independent mechanism allows ribosomal protein nuclear import for ribosome assembly”, which was published in eLife, Olga Schubert performed SRM-MS data acquisition, analysis and evaluation in the laboratory of Prof. R. Aebersold at the ETH Zurich. Initial importin binding assays were performed by Dr. Ute Fischer and myself in the laboratory of Prof. G. Schlenstedt at the University of Homburg and were aided by Dr. Stefanie Caesar and Silke Guthörl. I performed aggregation measurements at Novartis institute for biomedical research in Basel, in the laboratory of Dr. J. Ottl, supported by O. Esser, D. Barlier and R. Brunner. Dr. Ute Fischer performed importin binding assays and assisted with microscopic, biochemical and data analyses and also contributed to manuscript writing. Martin Altvater contributed to live cell imaging, indirect immunofluorescence microscopy, biochemical and data analysis. Dr. Yiming Chang assisted with biochemical analysis. Purnima Nerurkar, Cohue Peña and Michaela Gerber contributed to biochemical and cell biological analyses. I guided the project, performed all FISH experiments, sucrose gradient analyses and contributed essentially to cell biological and biochemical experiments and analyses and to manuscript writing. Prof. Vikram Panse headed the project, gave intellectual input and made significant contributions to the writing of the manuscript. Written parts, which are copied or adapted from my previous publications, are indicated.

I performed all biochemical and cell biological experiments of the second part “Mechanistic and structural insight into the role of the escortin Tsr2”. With the help and advise of Dr. Erich Michel from the laboratory of Prof. F. Allain, proteins for NMR spectroscopy were purified and NMR measurements and assignments were made. Dr. Erich Michel completed the backbone and side-chain assignments of Tsr21−152 and performed the structure calculations. Dr. Erich Michel also aided with the writing of the structural part, including materials and methods and results.
ABBREVIATIONS

AMP  Ampicillin
ARM  Armadillo
CBP  Calmodulin binding protein
CEN  Centromeric plasmid
cNLS Classical nuclear localization sequence
Cys  Cysteine
DBA  Diamond-Blackfan anemia
DMSO Dimethly sulfoxide
dNTP Deoxyribonucleotide triphosphate
DTT  Dithiothreitol
EDTA Ethylenediaminetetraacetic acid
EM  Electron microscopy
ES  Expansion segments
FDR  False discovery rate
FG  Phenylalanine-glycine
FISH Fluorescence in situ hybridization
GAP  GTPase activating protein
GEF  Guanine nucleotide exchange factor
HEAT  Huntington, elongation factor 3, PP2A, TOR
HetNOE  Heteronuclear NOE
HRP  Horseradish-peroxidase
HSQC  Heteronuclear single quantum coherence spectroscopy
IBB  Importin-β binding
IDA  Information-dependent acquisition
ITS1  Internal transcribed spacer 1
KAN  Kanamycin
KFS  Klippel-Feil syndrome
LB  Luria-Bertani
LDS  Lithium dodecyl sulphate
mRNA  Messenger RNA
mRNP  Messenger ribonucleoprotein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>NE</td>
<td>Nuclear envelope</td>
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<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
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<tr>
<td>NOESY</td>
<td>Nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
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<tr>
<td>NUP</td>
<td>Nucleoporin</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RanGAP</td>
<td>Ran GTPase activating protein</td>
</tr>
<tr>
<td>RanGEF</td>
<td>Ran guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>RG</td>
<td>Arginine-glycine</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root-mean-square deviation</td>
</tr>
<tr>
<td>RNA pol</td>
<td>RNA polymerase</td>
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<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
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<tr>
<td>r-protein</td>
<td>Ribosomal protein</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RVC</td>
<td>Ribonucleoside vanadyl complex</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic defined medium</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>snoRNA</td>
<td>Small nucleolar RNA</td>
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<tr>
<td>snoRNP</td>
<td>Small nucleolar ribonucleoprotein</td>
</tr>
<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SRM-MS</td>
<td>Selective reaction monitoring mass spectrometry</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>SSU</td>
<td>Small subunit</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-lineage acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCS</td>
<td>Treacher Collins syndrome</td>
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<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>TOCSY</td>
<td>Total correlation spectroscopy</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole cell extract</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose medium</td>
</tr>
<tr>
<td>5' TOP</td>
<td>5’ terminal oligopyrimidine tracts</td>
</tr>
<tr>
<td>5' UTR</td>
<td>5’ untranslated region in mRNA</td>
</tr>
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INTRODUCTION

1. NUCLEOCYTOPLASMIC TRANSPORT

The compartmentalized nature of eukaryotic cells ensures the separation of the genetic material in the nucleus from the surrounding cytoplasm. However, the nucleus is not completely isolated from the cytoplasm, since the bilayered nuclear envelope (NE) that surrounds it contains portals, called nuclear pore complexes (NPCs), which mediate nucleocytoplasmic transport events. Small molecules such as water, sugar, ions and small proteins can freely diffuse through the ~9 nm pore of the NPCs. However, the transport of bigger cargoes (>40 kDa) is highly selective and tightly regulated (Bonner, 1975; Paine et al., 1975). A highly specialized transport system has evolved that mediates bidirectional transport through the NPC. Specific transport signals present on cargoes contribute to the selectivity and specificity of transport processes (For review, see Xu et al., 2010).

Multiple types of nuclear transport events are required in eukaryotic cells. Proteins such as histones and components of the DNA polymerases are synthesized in the cytoplasm and must be imported into the nucleus. Messenger RNAs (mRNAs), transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), synthesized in the nucleus need to be exported into the cytoplasm, where they function in translation. Ribosome biogenesis involves multiple transport events through nuclear pore complexes. First, mRNAs encoding ribosomal proteins (r-proteins) are exported into the cytoplasm, where their translation occurs. Next, r-proteins are imported into the nucleolus/nucleus, where they assemble into rRNA containing ribosomal precursors. Finally, these assembled precursors are transported into the cytoplasm (For review, see Görlich and Kutay, 1999). In addition, nucleocytoplasmic shuttling proteins are continuously transported into and out of the nucleus.
1.1. Structure of the NPC

The NPC, connecting cytoplasm and nucleoplasm, has an octagonal symmetry along the nucleocyttoplasmic axis and a twofold axis perpendicular to it. Two coaxial rings, one in the nuclear periphery and one in the cytoplasmic periphery, are connected by eight elongated structures termed spokes (Unwin and Milligan, 1982). Eight fibrils extend from the cytoplasmic ring into the cytoplasm. A basket-like structure built by eight long filaments connected at their extremities extends into the nucleoplasm (Franke and Scheer, 1970; Goldberg and Allen, 1992; Jarnik and Aebi, 1991; Kiseleva et al., 2004).

Each individual NPC is composed of 30 evolutionarily conserved proteins termed nucleoporins, each present in multiple copies to form complexes of ~500-1000 polypeptides (Cronshaw et al., 2002; Davis and Blobel, 1986; DeGrasse et al., 2009; Rout et al., 2000). The NPC in yeast has a molecular mass of ~66 MDa (Rout and Blobel, 1993). Based on their localization in the NPC and on sequence features, nucleoporins are categorized as integral membrane proteins of the nuclear envelope, membrane apposed coat nucleoporins, linker nucleoporins, channel nucleoporins, nuclear basket nucleoporins or cytoplasmic filament nucleoporins (Figure 1) (Hoelz et al., 2011; Hsia et al., 2007). Most nucleoporins have a modular architecture that utilizes the following structural units: α-helical regions, β-propellers and unstructured phenylalanine-glycine (FG) repeats (Devos et al., 2006).

The integral membrane proteins of the NPC act as anchors for the entire complex and, together with the coat nucleoporins, form the core scaffold of the NPC (Devos et al., 2004; Rout et al., 2000). This core also contains linker nucleoporins that form interactions with all other symmetric nucleoporins (Fahrenkrog et al., 2000; Kosova et al., 2000; Lutzmann et al., 2002; Lutzmann et al., 2005; Schrader et al., 2008; Sinososoglou et al., 2000; Sinososoglou et al., 1996; Vollmer and Antonin, 2014). The innermost layer of the NPC channel, the central tube, is formed by the channel nucleoporins, which consist of an α-helical region flanked by FG-repeats (Grandi et al., 1993; Grandi et al., 1995; Wente et al., 1992). The α-helical regions of the
channel nucleoporins enclose the NPC channel, whereas the FG-repeats protrude into the central transport channel, thereby contributing to the permeability barrier. The nuclear basket nucleoporins and the cytoplasmic filament nucleoporins belong to the asymmetric nucleoporins, contributing to the directionality of nucleocytoplasmic transport (Ben-Efraim and Gerace, 2001; Ullman et al., 1999). Cytoplasmic filaments expose binding sites for mRNA export factors (Grandi et al., 1993; Grandi et al., 1995; Kosova et al., 1999; Zabel et al., 1996). The nuclear basket in yeast is composed of Nup1, Nup2, Nup60, Mlp1 and Mlp2. The nuclear basket component Nup2 seems to be responsible to disassemble the importin-α:cargo (Srp1:cargo in yeast) complex (Gilchrist et al., 2002; Hood et al., 2000; Solsbacher et al., 2000). An N-terminal sequence motif in Nup2 interacts with the nuclear localization sequence (NLS) binding site of importin-α, thereby releasing the NLS-containing cargo (Conti et al., 1998; Matsuura et al., 2003; Matsuura and Stewart, 2005).

Figure 1. Schematic model of the NPC. The NPC has an eight fold rotational symmetry and is composed of ~500-1,000 molecules of nucleoporins. Cytoplasmic filaments and nuclear basket nucleoporins function as docking sites for nuclear transport factors. FG-rich nucleoporins form the permeability barrier and mediate translocation events through the NPC. Nucleoporins of the nuclear envelope, coat and linker nucleoporins function in anchoring, shaping and tethering the NPC. From Tran et al., 2014.

1.2. Models of transport

Although the structure and function of the NPC have been extensively studied during the last decades, the mechanisms by which selectivity and speed of transport are achieved on a molecular level remain elusive. Several
models try to explain how the NPC achieves selective and rapid transport.

The Brownian affinity model suggests that FG-repeat containing nucleoporins lining the central transport channel prevent non-binding molecules from entering by thermal motion (Rout et al., 2003). Within the central tube of the NPC the movement of a molecule is highly restricted and its entropy is decreased. The bigger the cargo, the higher the entropic price that has to be paid to pass through the NPC. However, the enthalpy of binding of transport receptors to the FG-repeat-rich nucleoporins compensates the entropic penalty of penetrating through the NPC and facilitates the translocation process.

The selective phase model suggests that the FG-repeat containing nucleoporins interact by weak hydrophobic interactions to form a sieve-like meshwork with properties of a hydrogel (Frey and Görlich, 2007; Frey et al., 2006). Transport receptors move through this meshwork by locally dissolving the interactions between FG-repeats of nucleoporins (Figure 2A).

According to the forest model, FG-domains of nucleoporins can be classified into two groups (Yamada et al., 2010). One group adopts globular, collapsed-coil conformations (shrubs) whereas the other group adopts extended-coil confirmations (trees). A specific arrangement of “trees” and “shrubs” forms two separate zones of traffic with distinct physiochemical properties. FG-repeats with extended conformations transport nuclear transport receptors without or with small cargoes through a small channel in the middle of the NPC. Large cargoes bound to their transport receptors pass through the central zone lined by FG-repeats with collapsed-coil conformations (Figure 2B).

The reduction of dimensionality model implies that the FG-repeats of nucleoporins lining the central NPC channel are constantly saturated with nuclear transport receptors, leading to a permanent collapse of the FG-filaments (Peters, 2005). Interactions between FG-repeats and transport receptors create an inner tube for diffusion of small molecules. Transport receptor:cargo complexes interact with FG-repeats at the periphery of the NPC and follow a two-dimensional path along the NPC central channel. High concentrations of RanGTP at the channel exit release the complex from the FG-repeats and cause complex disassembly (Figure 2C).
1.3. Nuclear transport receptors

Nucleocytoplasmic transport is mainly mediated by importin-β-like transport receptors, which transiently interact with FG-repeats of nucleoporins. Most members of the importin-β-like transport receptors have similar domain architecture, including an N-terminal binding domain for the small GTPase Ran and a C-terminal cargo-binding domain (Figure 3; Chook and Blobel, 2001; Conti, 2002). Most importin-β-like transport receptors are restricted to either import or export. Yeast have ten importins required for nuclear import: Kap95, Kap114, Kap123, Kap104, Pse1, Pdr6, Sxm1, Mtr10, Nmd5, Kap120. In addition, three exportins Cse1, Crm1/Xpo1 and Los1 are
required for nuclear export and Msn5, an importin-β-like transport receptor, functions in both import and export. These importin-β-like transport receptors, their substrate specificities and human homologs are summarized in Table 1.

**Figure 3. Importin-β structure.**
(A) Kap95 (yeast) bound to RanGTP (dark grey, vertebrae) (Lee *et al.*, 2005). Kap95 is colored from blue (N-terminus) to red (C-terminus) and its 19 HEAT repeats are numbered as H1-H19 (Zachariae and Grubmuller, 2008). RanGTP binds to HEAT repeats 1-8. (B) Human importin-β (colored) bound to the IBB domain of importin-α (dark grey) (Cingolani *et al.*, 1999).

The fact that there are many more cargoes that need to be transported through the NPC than importin-β-like transport receptors suggests that each receptor transports multiple cargoes. Importin-β-like transport receptors are typically composed of approximately 20 HEAT repeats (Figure 3), each of which consists of two antiparallel α-helices, termed the A and B helices, connected by a short turn (Andrade *et al.*, 2001). Together, the HEAT repeats form an elongated helicoidal molecule (Figure 3) (Cingolani *et al.*, 1999; Lee *et al.*, 2005). HEAT repeats 1-8 interact with RanGTP, whereas HEAT repeats 7-19/20 contain binding sites for different transport substrates (Chook and Blobel, 1999). Importin-β (Kap95 in yeast) was the first identified member of the importin-β-like transport receptors. Together with its adaptor importin-α (Srp1 or Kap60 in yeast), importin-β mediates classical nuclear import (see Import of classical NLS containing cargoes) (Adam and Adam, 1994; Adam and Gerace, 1991; Enenkel *et al.*, 1995; Görlich *et al.*, 1994; Radu *et al.*, 1995).
Table 1. Yeast transport receptors.
ND = not defined, NC = not conserved. Adapted from Rothenbusch, 2012 and Chook and Blobel, 2001.

<table>
<thead>
<tr>
<th>S. cerevisiae</th>
<th>Cargo</th>
<th>NLS/NES</th>
<th>Human Homolog</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Import</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kap95</td>
<td>Many cargoes, including cargoes with basic, classical NLS (via Srp1/Importin-α) (Enenkel et al., 1995)</td>
<td>Classical NLS</td>
<td>Importin β1</td>
</tr>
<tr>
<td>Kap104</td>
<td>Nab2, Nab4 (1996), Hrp1 (Lange et al., 2008), Tfg2 (Suel and Chook, 2009)</td>
<td>PY-NLS, RG-NLS</td>
<td>Transportin, Importin β2</td>
</tr>
<tr>
<td>Mtr10/Kap111</td>
<td>Npl3 (Pemberton et al., 1997)</td>
<td>RG-NLS</td>
<td>Transportin SR1 and SR2</td>
</tr>
<tr>
<td>Kap123</td>
<td>Ribosomal proteins (Rout et al., 1997; Schlenstedt et al., 1997), histones H3 and H4 (Mosammaparast et al., 2002)</td>
<td>ND</td>
<td>Importin 4</td>
</tr>
<tr>
<td>Pse1/Kap121</td>
<td>Ribosomal proteins (Rout et al., 1997), histones H3 and H4 (Mosammaparast et al., 2002), Pho4 (Kaffman et al., 1998b), Aft1 (Ueta et al., 2003), Yap1 (Isoyama et al., 2001)</td>
<td>RG-NLS, lysine-rich NLS</td>
<td>Importin 5</td>
</tr>
<tr>
<td>Kap114</td>
<td>TBP (Morehouse et al., 1999; Pemberton et al., 1999), histones H2A, H2B (Mosammaparast et al., 2001), Sua7 (Hodges et al., 2005), Rpf1 (Caesar et al., 2006), Nap1 (Mosammaparast et al., 2002)</td>
<td>NC</td>
<td>Importin 9</td>
</tr>
<tr>
<td>Nmd5/Kap119</td>
<td>TFIIIs (Albertini et al., 1998), Hog1 (Ferrigno et al., 1998), Rpf1 (Caesar et al., 2006), Crz1 (Polizotto and Cyert, 2001)</td>
<td>ND</td>
<td>Importin 7</td>
</tr>
<tr>
<td>Sxm1/Kap108</td>
<td>Ribosomal proteins (Sydorskyy et al., 2003), Lhp1 (Rosenblum et al., 1997), Pab1 (Brune et al., 2005)</td>
<td>ND</td>
<td>Importin 8</td>
</tr>
<tr>
<td>Pdr6/Kap122</td>
<td>TFIIa (Toa1, Toa2) (Titov and Blobel, 1999)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kap120</td>
<td>Rpf1 (Caesar et al., 2006)</td>
<td>ND</td>
<td>Importin 11</td>
</tr>
<tr>
<td><strong>Export</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crm1/Xpo1</td>
<td>Proteins with leucine-rich NES, Yrb1 (Maurer et al., 2001), Hog1 (Ferrigno et al., 1998), Ssb1 (Shulga et al., 1999)</td>
<td>Leucine-rich NES</td>
<td>Crm1</td>
</tr>
<tr>
<td>Los1</td>
<td>tRNA (Hellmuth et al., 1998)</td>
<td>ND</td>
<td>Exportin-t</td>
</tr>
<tr>
<td>Cse1</td>
<td>Srp1/Kap60/Importin-α (Hood and Silver, 1998; Solsbacher et al., 1998)</td>
<td>ND</td>
<td>CAS</td>
</tr>
<tr>
<td><strong>Import/Export</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Msn5</td>
<td>Import: RPA (Yoshida and Blobel, 2001), Export: Pho4 (Kaffman et al., 1998a), Mig1 (DeVit and Johnston, 1999), Far1 (Blondel et al., 1999)</td>
<td>ND</td>
<td>Exportin 5</td>
</tr>
</tbody>
</table>
With the exceptions of Kap95 (Koepp et al., 1996), Crm1 (Stade et al., 1997), Cse1 (Xiao et al., 1993) and Pse1 (Seedorf and Silver, 1997), none of the yeast transport factors are essential since many of them have partially redundant functions and similar substrate specificities (Caesar et al., 2006; Mosammaparast et al., 2001; Seedorf and Silver, 1997). Significant similarities were identified between Kap95, Kap123, Pse1 and Kap104 (Aitchison et al., 1996). Kap123 is the most abundant of these four homologs (Ghaemmaghami et al., 2003) and localizes mainly to the cytoplasm and to the nuclear envelope, similar to Kap95. Kap123 is a 123 kDa large protein that binds specifically to the GTP bound form of Ran (Gsp1 in yeast, hereafter termed Ran). Kap123 does not import substrates with classical NLSs (see Import of classical NLS containing cargoes), but instead was shown to mediate transport of various r-proteins including uL23, eS1, eL8, eL18, uL11, eL32, uL5, eL42 (Rout et al., 1997) and uL23 (Schlenstedt et al., 1997) (nomenclature of r-proteins according to Ban et al., 2014; Table S1). Dividing yeast cells require the import of ~150,000 r-proteins per minute. Since deletion of KAP123 does not have a significant effect on cell growth it is evident that other importin-β-like transport receptors are implicated in transport of r-proteins. Pse1, Sxm1 and Nmd5 are suggested to functionally substitute Kap123 (Rout et al., 1997; Sydorskyy et al., 2003).

1.4. Role of the RanGTPase gradient in nucleocytoplasmic transport

Ran (Ras-related nuclear protein) belongs to the family of Ras-like GTPases. Ran is a very abundant, primarily nuclear localized protein, and, like other Ras family members, its interaction with other proteins depends on its nucleotide-bound state. Because Ran has a low intrinsic GTPase activity, its nucleotide-bound state is regulated by a GTPase activating protein (GAP) (Becker et al., 1995; Bischoff et al., 1994), which stimulates its GTPase activity and a GDP/GTP exchange factor (GEF) (Bischoff and Ponstingl, 1991).
Figure 4. The Ran (Gsp1) cycle in yeast.
The nucleotide exchange factor Prp20 (RCC1 in human) stimulates the nucleotide exchange of RanGDP to RanGTP in the nucleus. RanGTP disassembles importin:cargo complexes in the nucleoplasm and is exported to the cytoplasm bound to an importin or as a trimeric RanGTP:exportin:cargo complex. In the cytoplasm, Rna1 (RanGAP1 in humans) stimulates the GTP hydrolysis of Ran to disassemble the exported complexes. RanGDP has a high affinity for the heterodimeric transport receptor Ntf2 and is re-imported into the nucleus.

In addition to its nuclear functions (e.g. cell cycle control, DNA replication, etc.), Ran is the main regulator of the nucleocytoplasmic transport (Rush et al., 1996; Sazer, 1996). The characteristic feature of the Ran system driving nucleocytoplasmic transport is the asymmetric subcellular distribution of RanGTP and RanGDP. The RanGEF (RCC1, Prp20 in yeast) is chromatin bound and generates a high RanGTP concentration in the nucleus by exchanging GDP on Ran to GTP (Akhtar et al., 2001; Dilworth et al., 2005; Hahn et al., 2008; Nemergut et al., 2001; Ohtsubo et al., 1987). In contrast, the RanGAP (RanGAP1, Rna1 in yeast) is excluded from the nucleus and stimulates the GTPase activity of Ran in the cytoplasm (Becker et al., 1995; Bischoff et al., 1994). This GTP hydrolysis is the only energy-dependent process during the nucleocytoplasmic transport process (Englemeier et al., 1999; Ribbeck et al., 1999; Schwoebel et al., 1998). Consequently, a steep
gradient of RanGTP with high concentration in the nucleus and low concentration in the cytoplasm (Figure 4) and vice versa, high concentration of RanGDP in the cytoplasm and low concentration in the nucleoplasm is generated. This gradient is required for active transport but also for the directionality of the transport (Görlich et al., 2003).

Beside being the energy source for nuclear transport, RanGTP has a second role during the import process by mediating the import termination through direct interaction with importins, thereby dissociating the importin:cargo complex (Görlich and Mattaj, 1996; Rexach and Blobel, 1995). RanGTP is transported into the cytoplasm bound to an importin or as part of a trimeric export complex. After translocation through the NPC, RanGAP1/Rna1 stimulates together with its cofactor RanBP1 (Yrb1 in yeast) the GTP hydrolysis that triggers the complex dissociation. RanGDP has a high affinity to its transport receptor Ntf2 (Stewart et al., 1998; Wong et al., 1997), which translocates the complex through interactions with FG-rich nucleoporins back into the nucleoplasm (Figure 4) (Bayliss et al., 2002; Clarkson et al., 1996; Ribbeck et al., 1998).

1.5. Nuclear import

1.5.1. Import of classical NLS containing cargoes

The classical NLS (cNLS) was the first nuclear transport signal discovered and consists of two types: monopartite and bipartite. The SV40-type monopartite NLS is composed of a single stretch of 4-5 positively charged lysine and arginine residues (Kalderon et al., 1984), whereas the bipartite NLS consists of two basic clusters, separated by 10-12 variable amino acids (Dingwall and Laskey, 1991). The molecular recognition of a NLS within a cargo is central for the formation of the import complex and is mediated by specific sites on importin-α (Srp1 or Kap60 in yeast) (Conti and Kuriyan, 2000; Conti et al., 1998; Fontes et al., 2000). Importin-α is a highly conserved macromolecule composed of two structural domains. The short, basic N-terminal domain binds importin-β (IBB domain), whereas the C-terminus is composed of a tandem series of curved Armadillo (ARM) repeats,
which contain the NLS binding region (Figure 5). The major and minor NLS-binding sites are located at the concave site of importin-α and are formed by an array of tryptophan, asparagine and acidic residues. Monopartite NLSs generally bind to the major NLS-binding site, whereas bipartite NLS bind to both major and minor NLS-binding sites (Conti et al., 1998; Fontes et al., 2003).

![Figure 5. Structure of importin-α bound to a bipartite NLS.](image)
The ten Armadillo repeats are numbered as ARM1-10. Major and minor NLS binding sites are indicated. The Prp20 NLS, bound to importin-α, is shown in grey (Roman et al., 2013).

NLSs are usually located at unstructured loops on the surfaces of cargo proteins that conform to the importin-α binding site upon binding (Conti and Kuriyan, 2000; Conti et al., 1998; Lee et al., 2006). The IBB domain of importin-α contains a cluster of basic residues, similar to a cNLS, which can bind to its NLS-binding site and thereby compete with cargo NLS binding. The conformational change of importin-α upon binding to importin-β allows the interaction with the cNLS containing cargo, forming a ternary importin-β:importin-α:cargo complex. The IBB domain binds mainly as a long helix to the C-terminal part of importin-β along the B helices of HEAT repeats 7-19 (Cingolani et al., 1999) and is deeply embedded in a tight spiral (Figure 3B). Cargo recognition by importin-α is distinct from the mechanism by which non-classical NLSs are recognized by importin-β-like transport receptors (see Non-classical nuclear import).
FG-repeat containing nucleoporins are supposed to mediate the transport of cargo:receptor complexes through the NPCs. The hydrophobic phenylalanine side-chains of the FG-repeat containing nucleoporins interact weakly with hydrophobic patches on the surface of the transport receptors. These weak and transient interactions permit rapid transport of cargo:receptor complexes (Bayliss et al., 2000a; Bayliss et al., 2002; Bayliss et al., 2000b; Bayliss et al., 1999; Fribourg et al., 2001; Grant et al., 2003; Liu and Stewart, 2005; Rexach and Blobel, 1995; Ribbeck and Görlich, 2002). The number of FG-repeats and the linker regions between the FG-repeat cores are proposed to contribute to binding, perhaps by modulating the strength of the interaction (Liu and Stewart, 2005; Pyhtila and Rexach, 2003). Binding affinities between importin-β:importin-α:cargo complexes and FG-rich nucleoporins have been proposed to increase from the cytoplasmic to the nuclear face of the NPC, providing a possible additional driving force to the RanGTP gradient which powers nuclear protein import (Ben-Efraim and Gerace, 2001).

Upon arrival in the nucleoplasm, RanGTP binding to importin-β dissociates the importin-α:importin-β complex and releases the importin-α:cargo complex (Figure 6). Since the RanGTP and the IBB-domain binding sites on importin-β only partially overlap, RanGTP releases the IBB domain mainly by inducing a conformational change in importin-β (Lee et al., 2005; Vetter et al., 1999). Interaction of importin-α with the FG-repeats of Nup50 (yeast Nup2) finally releases the cargo from the Importin-α:cargo complex (Matsuura and Stewart, 2005; Rexach and Blobel, 1995; Solsbacher et al., 2000). After import complex disassembly, importin-α and importin-β are separately recycled back to the cytoplasm. Importin-β travels to the cytoplasm in complex with RanGTP (Izaurralde et al., 1997). Upon arrival in the cytoplasm, Rna1/RanGAP1, together with the cofactor Yrb1/RanBP1, binds to the complex and stimulates the GTP hydrolysis of RanGTP to RanGDP (Figure 6) (Bischoff and Görlich, 1997). In contrast, importin-α is exported by the exportin Cse1/CAS, in a trimeric CAS:importin-α:RanGTP complex. GTP hydrolysis of RanGTP dissociates the trimeric complex and recycles importin-α for new round of nuclear import (Figure 6) (Kutay et al., 1997a).
Figure 6. Nuclear import cycles.
Classical importin-α mediated import is shown in the upper pathway. Cargoes containing a classical basic NLS interact with the transport receptor importin-β via importin-α. In the nucleoplasm, RanGTP disassembles the complex through interaction with importin-β. Nuclear import mediated directly by importin-β-like transport receptors (here e.g. for importin-β, see Non-classical nuclear import) is shown in the lower pathway. Importin:RanGTP complexes are recycled back to the cytoplasm, where GTP hydrolysis triggered by Rna1 releases the importin for new import cycles.

1.5.2. Non-classical nuclear import

Non-classical nuclear import relies on the direct interaction between nuclear localization sequences in cargoes and importin-β-like transport receptors. These NLSs are highly diverse, encompassing different structural domains and linear epitopes, and show little sequence or structural similarities. However, most cargoes bind to the inner concave site of importins. Snurportin 1, for instance, an adaptor that recognizes m3G-Caps in uridyl-rich small nuclear ribonucleoproteins (snRNPs), contains an IBB domain similar to importin-α, which becomes embedded within the B helices
of HEAT repeats 7-19 and interacts in a mainly electrostatic manner (Mitrousis et al., 2008). SREBP-2 binds directly to importin-β between HEAT repeats 7-19 via hydrophobic interactions and is enclosed by two helices from HEAT repeats 7 and 17 (Lee et al., 2003). The mammalian homolog of the importin Kap104, Kapβ2, binds directly to ~20 different mRNA binding proteins (Bonifaci et al., 1997; Pollard et al., 1996). Recently, several common characteristics between Kapβ2/Kap104 cargo signals were identified, termed PY-NLS (Lange et al., 2008; Lee et al., 2006). PY-NLS consensus motifs consist of a hydrophobic or basic N-terminal motif and a RX_{2,5}PY motif on the C-terminus (Cansizoglu et al., 2007). PY-NLSs usually adopt extended conformations lining a common interface on the C-terminal arch of Kapβ2 through their interactions with different acidic or hydrophobic patches, respectively.

Similarly to the classical import pathway, importin:cargo complexes translocate through the NPC into the nucleus, where RanGTP binding triggers cargo release. The main trigger for cargo release is a structural rearrangement in the importin upon RanGTP binding (Figure 6). However, some cargo binding sites completely overlap with the RanGTP binding site, making RanGTP and cargo binding mutually exclusive. In Kapβ2, RanGTP also binds along the concave surface of its N-terminal arch (Chook and Blobel, 1999). RanGTP binding to Kapβ2 causes structural rearrangements in an acidic loop, involved in RanGTP binding, to occur, shifting the loop towards the NLS binding site, where it repels the cargo and triggers release (Chook et al., 2002).

1.6. Nuclear export

Nuclear export occurs upon formation of a trimeric complex between an exportin, a cargo and RanGTP. Through interactions with the FG-repeats of nucleoporins, the export receptor mediates the translocation of the export complex into the cytoplasm. Upon arrival in the cytoplasm, either cytoplasmic RanBP1 (Coutavas et al., 1993; Ouspenski et al., 1995) or RanBP2 (Walther et al., 2002; Wu et al., 1995; Yokoyama et al., 1995), a component of the
nuclear pore, binds to the exportin:cargo:RanGTP complex. This interaction weakens the export complex, rendering RanGTP accessible to RanGAP1 (Bischoff et al., 2002). Yrb1, the yeast homolog of RanBP1, facilitates exportin:RanGTP complex disassembly (Schlenstedt et al., 1995). After dissociation, the exportin travels in its unbound state back to the nucleoplasm (Figure 7) (Kutay et al., 1997b).

![Figure 7. Nuclear export cycle in yeast.](image)

A trimeric export complex composed of exportin, RanGTP and cargo is formed in the nucleoplasm. The exportin interacts with FG-rich nucleoporins and translocates the export complex through the NPC. In the cytoplasm, Rna1, together with its cofactor Yrb1, stimulates the GTPase activity of Ran. After complex dissociation, the exportin travels back to the nucleoplasm.

Exportins recognize specific signals, termed nuclear export sequences (NESs) in their cargoes. The best characterized NES is the hydrophobic, leucine-rich NES, which is composed of four or five hydrophobic (often leucine) residues separated by variable amino acids (Güttler et al., 2010). Hydrophobic NESs are recognized by the export receptor Crm1 (Fornerod et al., 1997; Stade et al., 1997). Crm1 contributes to the export of various classes of RNAs and ribonucleoproteins. However, since Crm1 is not able to bind RNA directly, NES-containing adaptor proteins are required to couple the different cargoes to its export receptor. The Crm1-mediated export of different cargoes additionally requires the nuclear cofactor RanBP3 (Yrb2 in yeast) (Mueller et al., 1998; Noguchi et al., 1997; Taura et al., 1997). RanBP3 also
binds to Crm1, forming a quaternary complex with RanGTP and cargo, and is thought to regulate the affinity of Crm1 to different export substrates (Englmeier et al., 2001; Lindsay et al., 2001). Crm1 is unusual in its broad range of cargoes. For example, the only substrate known for Cse1/CAS is Srp1/importin-α, which needs to be recycled to the cytoplasm in context of the classical import pathway. In addition, the transport receptor Msn5 specifically recognizes phosphorylated export substrates, whereas Los1 is dedicated to tRNA export.

1.7. Ribosomes in nucleocytoplasmic transport

The production of ribosomes accounts for the major fraction of nucleocytoplasmic transport in growing yeast cells (Rout et al., 1997; Sydorskyy et al., 2003), and requires both the nuclear import and export machinery. Since translation of all proteins occurs in the cytoplasm, mRNAs encoding r-proteins first need to be exported from the nucleoplasm to the cytoplasm. These r-proteins as well as required trans-acting biogenesis factors are then imported into the nucleolus/nucleus, where the main steps of ribosome assembly and maturation occur. After nuclear maturation, pre-ribosomes are exported into the cytoplasm, where shuttling trans-acting factors are released and recycled back into the nucleoplasm. Therefore multiple rounds of nucleocytoplasmic transport are critical for ribosome biogenesis.
2. RIBOSOME BIOGENESIS

Ribosomes are the molecular factories responsible for decoding genetic information into proteins, making the assembly of functional ribosomes a fundamental process for all living cells. Eukaryotic ribosomes are composed of two subunits: small (40S) and large (60S). The small ribosomal subunit is responsible for decoding the genetic information by matching tRNAs with mRNA codons, whereas the large ribosomal subunit carries out the peptidyl transfer reaction required to synthesize polypeptide chains.

2.1. The structure of the mature ribosome

The eukaryotic small 40S subunit is composed of the 18S rRNA and 33 r-proteins (Figure 8). The different domains of the 40S subunit form the characteristic hallmarks of its structure: the head, platform, body, beak, shoulder, right foot and left foot (Figure 10). The large 60S subunit is composed of 25S, 5S, 5.8S rRNA and 46 r-proteins (Figure 9). The key hallmarks of the large subunit are conserved from prokaryotes to eukaryotes, including the central protuberance, two stalks and the sarcin-ricin loop (Figure 10) (Ben-Shem et al., 2011; Klinge et al., 2011; Rabl et al., 2011).

Figure 8. R-proteins of the 40S subunit of T. thermophila.
R-proteins conserved across all kingdoms of life are colored in light blue, r-proteins found in both archea and eukaryotes are colored in orange and eukaryotic-specific r-proteins or extensions in red. From Rabl et al., 2011.
Figure 9. R-proteins of the 60S subunit of *T. thermophila*. R-proteins conserved across all kingdoms of life are colored in light blue, archael r-proteins in orange and eukaryotic-specific r-proteins or extensions in red. From Klinge *et al.*, 2011.

The 40S and 60S subunits form together the eukaryotic 80S ribosome. Eukaryotic ribosomes are, in contrast to their prokaryotic counterparts, more complex and much larger with a molecular mass of ~3.3 MDa (yeast), and contain additional r-proteins and rRNA stretches. These additional rRNA nucleotides form so-called expansion segments (ES) (Gerbi, 1996; Spahn *et al.*, 2001), which basically add an additional layer to the ribosome. Beyond the 79 r-proteins in the yeast ribosome, only six r-proteins of the 40S and six r-proteins of the 60S subunit are specific to eukaryotes (Lecompte *et al.*, 2002). However, many of the conserved r-proteins contain eukaryotic-specific extensions (Figure 8 and Figure 9) (Ben-Shem *et al.*, 2010; Rabl *et al.*, 2011). All r-proteins are involved in a tight network of protein-protein and protein-RNA interactions, forming a large interaction surface. Most r-proteins are composed of one globular domain generally located on the ribosome surface, from which long N- and/or C-terminal tails extend. These tails, rich in basic amino acids, are often buried within the rRNA framework and involved in rRNA interactions (Rabl *et al.*, 2011). Long r-protein extensions mediate long-range interactions and intersubunit contacts (Klinge *et al.*, 2011).
Figure 10. Comparison of prokaryotic and eukaryotic ribosomes. Prokaryotic ribosomal subunits are colored in blue and grey. R-proteins conserved across all kingdoms of life are colored in light blue, archael r-proteins in orange and eukaryotic-specific r-proteins or extensions in red. (A) and (C) Solvent exposed site and subunit interface of the 30S subunit of *Thermus thermophiles*, respectively (Selmer et al., 2006). (B) and (D) Solvent exposed site and subunit interface of the 40S subunit of *Tetrahymena thermophila*, respectively (Rabl et al., 2011) (E) and (G) Solvent exposed site and subunit interface of the 50S subunit of *T. thermophiles*, respectively (Selmer et al., 2006). (F) and (H) Solvent exposed site and subunit interface of the 60S subunit of *T. thermophila*, respectively (Rabl et al., 2011). Hallmarks of the subunits are indicated: H=head, Be=beak, Pt=platform, Sh=shoulder, Bo=body, LF=left foot, RF=right foot, CP=central protuberance, SRL=sarcin-rinic loop. A-, P-, E- sites and the exit tunnel are indicated. From Klinge et al., 2012.
Eukaryotic-specific r-proteins are mainly located at the subunit sides facing away from the subunit interface (Figure 10) (Klinge et al., 2011; Rabl et al., 2011), thereby offering many binding platforms for the more than 200 trans-acting factors crucial for eukaryotic ribosome biogenesis. Moreover, eukaryotic-specific r-proteins often bridge RNA ES, thereby forming secondary structures and anchoring ESs on the surface.

2.2. Ribosome biogenesis in budding yeast

Although the structure and function of the ribosome are well-established, many steps during the assembly process of these large molecular machines remain elusive. To build a ribosome, eukaryotic cells need to coordinate the assembly of more than 70 r-proteins with four different rRNA species. In order to ensure the high efficiency and accuracy of ribosome biogenesis, the concerted action of all three transcription machineries (RNA polymerases I, II and III) is also required (Tschochner and Hurt, 2003).

In contrast to prokaryotes, eukaryotic ribosome assembly is additionally aided by >200 non-ribosomal trans-acting factors (Fromont-Racine et al., 2003; Henras et al., 2008; Kressler et al., 1999). The highly coordinated multi-step process of ribosome biogenesis starts within the nucleolus with the formation of the pre-ribosomal 90S particle (Trapman et al., 1975; Udem and Warner, 1973). The 90S particle is then processed to yield the precursors of the 60S and 40S subunits, which are channeled into distinct biogenesis pathways. After nucleolar assembly, pre-60S and pre-40S particles undergo various maturation and processing events within the nucleoplasm before they become export competent and are transported through the NPC into the cytoplasm (Figure 11). Nuclear export of pre-ribosomal particles is dependent on the importin-β-like transport receptor Crm1 and a functional RanGTPase system (Moy and Silver, 1999, 2002). Pre-ribosomal particles arrive in the cytoplasm in a functionally inactive state and must undergo final cytoplasmic maturation steps to become translation competent. Emerging evidence indicates that nuclear and cytoplasmic maturation steps are closely coupled to nuclear export and final cytoplasmic proofreading of the ribosome.
Figure 11. Overview of ribosome biogenesis.

(A) Eukaryotic ribosome biogenesis. R-proteins and assembly factors are co-transcriptionally recruited to the 35S pre-rRNA to form the 90S pre-ribosome (dark blue). Cleavage at the A2 site separates the pre-40S (green) from the pre-60S (blue) maturation pathway. Assembly factors transiently interact with the precursors of both subunits and contribute to processing and maturation. The pre-40S subunit undergoes relatively few maturation steps in the nucleoplasm before it is exported into the cytoplasm. In the cytoplasm, a final rRNA processing event and a structural rearrangement occur. In contrast, the pre-60S subunit undergoes multiple maturation and processing steps in the nucleoplasm prior export into the cytoplasm.

(B) rRNA processing during ribosome biogenesis. An important prerequisite for ribosome biogenesis is the correct rRNA processing. The 35S pre-rRNA is processed at the 3' and 5' ends (Kufel et al., 1999) before A2 site cleavage is triggered by an unknown endonuclease that separates the 40S and 60S biogenesis pathways. The pre-40S subunit containing 20S pre-rRNA is exported into the cytoplasm where the endonuclease Nob1, aided by other trans-acting factors, cleaves the 20S pre-rRNA to mature 18S rRNA (Lamanna and Karbstein, 2009; Pertschy et al., 2009). The 27SA2 pre-rRNA can be processed via two pathways to yield different 5.8S rRNAs (Lygerou et al., 1996). Processing of 6S pre-rRNA is performed in the cytoplasm. Adapted from Gerhardy et al., 2014.

2.2.1. Assembly of the earliest 90S pre-ribosome

Ribosome biogenesis in S. cerevisiae starts with RNA polymerase I driven transcription of ribosomal DNA (rDNA) repeats in the nucleolus. The yeast nucleolus forms around ~150 tandem repeats of rDNA, present on chromosome XII. Since yeast cells must produce ~200,000 ribosomes per generation time, rDNA transcription has to be highly efficient (French et al., 2003; Kos and Tollervey, 2010).

The transcribed 35S pre-rRNA is the common precursor for mature 18S, 5.8S and 25S rRNA but also contains RNA sequences that are cleaved and so are not part of the mature ribosome. The 35S pre-rRNA interacts co-transcriptionally with r-proteins, mainly from the small 40S subunit, assembly factors and small nucleolar RNAs (snoRNAs) to form the 90S pre-ribosome (Trapman et al., 1975; Udem and Warner, 1972). The small subunit (SSU) processome, a RNP complex within the 90S particle, is required for the processing, assembly and maturation of the 40S subunit. The SSU processome consists of a huge U3 snoRNP, which contains many U3 snoRNA, U3-binding proteins, the proteins Utp1-17 and various additional proteins (Dragon et al., 2002) (reviewed in Phipps et al., 2011). The SSU processome assembles co-transcriptionally onto the 5’ end of the nascent rRNA and is necessary for early A0 and A1 pre-rRNA cleavage and early pre-40S assembly (Borovjagin and Gerbi, 1999; Dragon et al., 2002; Gallagher et al., 2004; Marmier-Gourrier et al., 2011). The stepwise assembly of
components of the SSU processome is coupled to pre-rRNA modification and processing and has been proposed to drive compaction of the 90S pre-ribosome (Perez-Fernandez et al., 2011). Two main classes of snoRNPs, box H/ACA snoRNPs and box C/D snoRNPs, are involved in pseudouridylation and 2’-O-ribose methylation, respectively (Kiss, 2001), contributing to 90S unfolding and compaction (Watkins and Bohnsack, 2012). Moreover, more than 60 different snoRNPs mediate >100 covalent modifications of the nascent rRNA during 90S assembly (Decatur and Fournier, 2002, 2003; Decatur et al., 2007; Hughes, 1996; Hughes and Ares, 1991). Co-transcriptional cleavage of the 35S pre-rRNA at site A₂ in the internal transcribed spacer 1 (ITS1) region finally separates the pre-40S subunit from the pre-60S subunit (Figure 11B) (Kos and Tollervey, 2010; Osheim et al., 2004). However, the endonuclease responsible for this cleavage step remains to be identified.

### 2.2.2. Nucleoplasmic maturation of pre-ribosomal subunits

#### 2.2.2.1. Nucleoplasmic maturation of the pre-40S subunit

After cleavage at sites A₀, A₁ and A₂, which generates 20S pre-rRNA, the composition of the pre-40S subunit changes dramatically. Many components of the SSU processome are rapidly released after pre-40S, pre-60S separation. Most r-protein of the small subunit are already present within these pre-40S particles (Ferreira-Cerca et al., 2007). R-proteins are important, not only for ribosomal function, but also for early nucleolar maturation events (uS13) (Leger-Silvestre et al., 2004) or for nuclear export (uS2, uS5, uS3, eS10, uS19) (Ferreira-Cerca et al., 2005). They also assist in rRNA folding, trigger conformational changes in pre-rRNA and form binding platforms for assembly and translation factors.

Until now, only a few trans-acting factors are known to be present on the nucleoplasmic pre-40S subunit. Many of these factors are already present on 90S pre-ribosomes (e.g. Enp1, Dim1, Mex67/Mtr2 and Rrp12) (Faza et al., 2012; Grandi et al., 2002), whereas other trans-acting factors like Nob1, Rio2, Ltv1 and Tsr1 bind after A₀-A₂ cleavage to the pre-40S subunit (Schäfer et al., 2003). These factors stay stably associated to the pre-40S upon nuclear
export and contribute to final cytoplasmic maturation of 40S subunits. Except for nuclear export factors (see Nuclear export of pre-ribosomes), the nuclear function of many of these factors is not well understood. Energy consuming enzymes like the kinase Hrr25 and the kinases/ATPases Rio1 and Rio2 are thought to prepare pre-40S subunits for nuclear export and/or cytoplasmic maturation (Geerlings et al., 2003; Vanrobays et al., 2003).

During their travel through the nucleoplasm, pre-40S subunits undergo only a few compositional changes. Nuclear pre-40S subunits already display most of the typical hallmarks of the mature 40S subunit including head, platform and body but they lack the characteristic beak structure (Schäfer et al., 2006). Close to the prospective 40S beak structure, a trimeric complex composed of Enp1, Ltv1 and the r-protein uS3 is formed. Phosphorylation of Enp1 and uS3 is suggested to increase the conformational flexibility and allow efficient nuclear export of pre-40S subunits (Schäfer et al., 2006).

2.2.2.2. **Nucleoplasmic maturation of the pre-60S subunit**

In contrast to pre-40S subunits, pre-60S subunits interact in the nucleoplasm with more than 150 assembly factors and undergo various maturation and processing events prior to export to the cytoplasm (Nissan et al., 2002). These factors bind to pre-ribosomal particles at distinct maturation steps and are released from particles, after having performed their assigned tasks, to be recycled back to the nucleus and participate in new rounds of maturation.

An important step in the nuclear pre-60S maturation pathway is the incorporation of 5S rRNA, produced by RNA Pol I. 5S rRNA is incorporated into the pre-60S subunit as a trimeric complex with the r-proteins uL18 (yeast Rpl5, Table S1) and uL5 (yeast Rpl11). uL18 and uL5 are co-imported into the nucleus by the importin Kap104 via the transport adaptor Syo1 (Kressler et al., 2012a). Once in the nucleus, RanGTP disassembles the import complex, and uL18 and uL5 are loaded onto the 5S rRNA. The assembly factors Rpf2 and Rrs1 aid the incorporation of the trimeric uL18:uL5:5S rRNA complex into the pre-60S particle (Ciganda and Williams, 2011; Zhang et al., 2007).
Multiple energy-consuming factors are involved in the assembly and release of trans-acting factors and determine the directionality of the maturation process. The AAA-ATPase Rix7, which is closely related to Cdc48 (p97 in mammals), uses ATP hydrolysis to strip off the assembly factor Nsa1 and thereby promotes the nucleolar to nucleoplasmic transition of the pre-60S subunit (Kressler et al., 2008). Rea1, the largest protein in yeast, consists of six ATPase domains and functions multiple times during nucleoplasmic maturation of the pre-60S subunit by triggering the ATP-dependent release of assembly factors (Bassler et al., 2010; Kressler et al., 2012b; Ulbrich et al., 2009). Together with the GTPase Nug2, Rea1 prevents premature formation of an export competent pre-60S subunit, thereby establishing a nuclear checkpoint (Matsuo et al., 2014). The pre-60S binding site for Nug2 overlaps with the binding site for the export adaptor Nmd3. Nug2 release from pre-60S subunits and subsequent export depends on its GTPase activity as well as on the ATPase activity of Rea1. This mechanism guarantees that only particles having undergone complete nucleoplasmic maturation are exported to the cytoplasm (Matsuo et al., 2014).

2.2.3. Nuclear export of pre-ribosomes

Translocation of the charged >2 MDa pre-ribosomal particles from the nucleoplasm to the cytoplasm through the NPC poses a major challenge for eukaryotic cells. It is estimated that, in a growing yeast cell, each NPC exports ~25 pre-ribosomal particles per minute to the cytoplasm (Warner, 1999). This rapid process requires an efficient and coordinated interplay between ribosomal trans-acting factors, components of the NPC, the transport receptor Crm1 and the RanGTPase cycle (Hurt et al., 1999; Stage-Zimmermann et al., 2000). The essential NES-containing Crm1 adaptor Nmd3 facilitates export of the pre-60S subunit by forming a trimeric complex with Crm1 and Ran (Figure 12) (Gadal et al., 2001; Ho et al., 2000). To ensure the efficient translocation of pre-ribosomes through the NPC, multiple export factors are required (Oeffinger et al., 2004). The HEAT-repeat containing protein Rrp12 (Oeffinger et al., 2004) and the essential heterodimeric mRNA export factor Mex67-Mtr2 (Faza et al., 2012; Santos-Rosa et al., 1998; Segref et al., 1997; Tuck and Tollervey,
2013; Yao et al., 2007) contribute to export of both pre-40S and pre-60S subunits. Both Rrp12 and the Mex67-Mtr2 heterodimer interact directly with FG-repeat containing nucleoporins and are thought to facilitate translocation through the NPC (Strasser et al., 2000; Strawn et al., 2001). The Mex67-Mtr2 heterodimer does not directly rely on the RanGTP gradients (Yao et al., 2007). Additionally, multiple non-essential auxiliary factors that interact with FG-repeat-rich nucleoporins facilitate the translocation of pre-ribosomes through the NPC. Only a few factors have been described to mediate pre-40S subunit export. In yeast, the two non-essential NES containing proteins Ltv1 and Dim2 are proposed to be Crm1 adaptors (Merwin et al., 2014; Seiser et al., 2006; Vanrobays et al., 2008). In mammalian cells, Rio2, in addition to Ltv1 and Dim2, is described to recruit Crm1 and accelerate the pre-40S export process. Although Rio2 is an essential protein, its NES is not absolutely required for Rio2 function (Zemp et al., 2009). Hence, an essential NES-containing Crm1 adaptor for pre-40S subunit export remains elusive, suggesting that multiple NES containing adaptors have redundant roles in recruiting Crm1. Efficient export of the small pre-40S subunit in yeast also requires the conserved Ran-binding protein Yrb2 (Moy and Silver, 2002; Taura et al., 1998). Yrb2 is not essential; however, yrb2Δ cells are slow growing at low temperatures and defective in pre-40S subunit nuclear export. The mammalian homolog of Yrb2, RanBP3, facilitates the loading of Crm1 and RanGTP to certain cargoes, thereby enabling nuclear export (Englmeier et al., 2001). Similarly, Yrb2 might deliver RanGTP and Crm1 to yet unknown NES-containing adaptors to mediate pre-40S nuclear export.

Multiple factors are known to mediate pre-60S subunit export (Figure 12). The trans-acting factor Arx1 binds to the ribosome exit tunnel and arrests the conserved rRNA ES27 in a tunnel conformation (Bradatsch et al., 2012; Greber et al., 2012). However, the mechanism by which Arx1 facilitates translocation of the pre-60S subunit through the NPC remains unstudied. Functional screens, designed to identify additional factors involved in nuclear export of pre-60S subunits, uncovered the shuttling trans-acting factors Bud20 and Ecm1 as well as the mRNA export factor Npl3 (Altvater et al., 2012; Bassler et al., 2001; Hackmann et al., 2011; Yao et al., 2010). In addition to
the FG-repeat binding transport factors, the non-FG-repeat interacting transport factor Gle2 was recently identified as a pre-60S export factor (Occhipinti et al., 2013). Gle2 binds directly to the Gle2-binding motif of the nucleoporin Nup116. Upon tethering to Nup116, Gle2 recruits the pre-60S subunit via a second binding site. Despite their identification, it remains unclear how these export factors work together to mediate nuclear export.

**Figure 12. Nuclear export of pre-ribosomes.**
Nuclear export of pre-ribosomes depends on the RanGTP gradient and on the export receptor Crm1. Many other assembly factors aid nuclear export by concomitant binding to pre-ribosomes and components of the NPC. Adapted from Gerardy et al., 2014.

### 2.2.4. Cytoplasmic maturation of pre-ribosomes

#### 2.2.4.1. Cytoplasmic maturation of pre-40S subunits

Upon arrival in the cytoplasm, the pre-40S undergoes two major events: First, a structural rearrangement occurs to generate the beak structure of the mature 40S subunit. Second, a final endonucleolytic cleavage of the 20S pre-rRNA occurs to yield mature 18S rRNA (Figure 11B). Dephosphorylation of Enp1 and/or uS3 is necessary for the stable incorporation of uS3 into the 40S subunit and crucial for the correct formation of the beak structure in the 40S subunit (Schäfer et al., 2006).

Dim1 dimethylates two consecutive conserved adenines at the 3’ end of the 18S rRNA (Lafontaine et al., 1994; Lafontaine et al., 1995; Udem and Warner, 1973). Although Dim1 methylation of the 20S pre-rRNA occurs in the cytoplasm, Dim1 is associated with the 90S pre-ribosome in the nucleolus and is required for early nucleolar processing events (Lafontaine et al., 1995;
Lafontaine et al., 1998; Schäfer et al., 2003). However, Dim1 dimethylation is not essential for 20S pre-rRNA processing, and is instead suggested to be involved in fine-tuning of translation (Lafontaine et al., 1998). Dim2 (Pno1) is also involved in early nucleolar cleavage events (Peng et al., 2003; Senapin et al., 2003; Vanrobays et al., 2004) and was suggested to recruit Dim1 to the 90S pre-ribosome (Vanrobays et al., 2004). However, recent studies suggest that Pno1 is involved in preventing premature translation initiation by preventing the binding of elF3 (Strunk et al., 2011). After dimethylation of the 20S pre-rRNA, the endonucleolytic cleavage of the 20S pre-rRNA renders the pre-40S subunit translation competent.

Multiple energy-consuming factors are required for efficient 20S pre-rRNA to 18S rRNA processing. In addition to the PIN-domain endonuclease Nob1, the helicase Prp43 and its cofactor Pfa1 (Pertschy et al., 2009), the kinases Rio1 and Rio2, and Tsr1 and Tsr2 are implicated in this process (Gelperin et al., 2001; Leger-Silvestre et al., 2004; Peng et al., 2003). Nob1 joins the pre-40S particle already in the nucleus; however, D-site cleavage of 20S pre-rRNA occurs only in the cytoplasm (Lamanna and Karbstein, 2009; Pertschy et al., 2009).

The GTPase Fun12 (eIF5B) promotes subunit joining of mature 60S subunits with immature pre-40S subunits, thereby forming 80S-like particles (Figure 13). This translation-like cycle triggers 20S pre-rRNA to 18S rRNA processing by Nob1 (Lebaron et al., 2012; Strunk et al., 2012). The ABC-type ATPase Rli1, aided by the tRNA mimic Dom34 (Becker et al., 2012) dissociates 80S-like particles, thereby releasing the endonuclease Nob1 (Strunk et al., 2012).

In addition to trans-acting factors, r-proteins are also important for cytoplasmic 40S subunit biogenesis. The r-proteins uS2, uS5, uS3, eS10, uS11, uS10 and eS21 (Table S1) have been shown to be required for 20S pre-rRNA cleavage (Ferreira-Cerca et al., 2005). uS11 (yeast Rps14), for example, localizes to the proximity of the 3’ end of the 18S rRNA (Rabl et al., 2011; Spahn et al., 2001) where it has been proposed to induce conformational changes in the 20S pre-rRNA that allow cleavage to occur. uS11 directly
interacts with the endonuclease Fap7, which is involved in 20S pre-rRNA processing (Granneman et al., 2005).

Figure 13. A translation-like cycle is responsible for final 40S maturation. (A) Translation-competent 40S subunits (green) containing mature 18S rRNA assemble with translation initiation factors (light red), mRNA and the initiator tRNA to form the pre-initiation complex. The GTPase and translation initiation factor eIF5b (Fun12) (red) promotes joining of the 60S subunit (light blue) and thereby initiates translation. After completion of translation, the ABC-type ATPase Rli1 (dark blue) dissociates 80S particles, thereby allowing reinitiation of translation. A= tRNA binding site; P= site of peptide bond formation; E= exit site. (B) Final 40S maturation steps involve formation of translation-like particles. Ribosome biogenesis factors (yellow) bound to pre-40S subunits containing 20S pre-rRNA prevent formation of the pre-initiation complex and translation. The GTPase and translation initiation factor eIF5b (Fun12) promotes the joining of mature 60S subunits and pre-40S particles, resulting in the formation of 80S-like particles and thereby stimulating the endonucleolytic cleavage of 20S pre-rRNA to 18S rRNA by Nob1. Similar to its role during translation termination, Rli1 dissociates these 80S-like particles and allows translation initiation to occur. From Schütz and Panse, 2012.

2.2.4.2. Cytoplasmic maturation of pre-60S subunits

Following nuclear export, multiple trans-acting factors need to be released from pre-60S subunits and recycled back to the nucleus to participate in further rounds of pre-60S maturation (Figure 14). The energy-consuming enzymes catalyzing these releasing events are the AAA-ATPase Drg1 and the ATPases Kre35 and Ef11, aided by their cofactors Sdo1, Jj1 and Rei1 (Panse and Johnson, 2010). Drg1 triggers the first cytoplasmic releasing event. Since pre-60S maturation factors are released sequentially in the cytoplasm, Drg1-dependent release is a prerequisite for all following maturation events. The Johnson Lab defined a pathway of cytoplasmic maturation by ordering the
known steps in cytoplasmic pre-60S maturation (Figure 14) (Lo et al., 2010). Rlp24 is the direct substrate of Drg1 and its release is necessary to allow incorporation of the r-protein eL24 (yeast Rpl24) (Kappel et al., 2012). eL24 incorporation triggers the recruitment of the zinc-finger proteins Yvh1 and Rei1 (Altvater et al., 2012; Lo et al., 2009). Rei1 is required for the recycling of the pre-60S export factor Arx1. Yvh1, on the other hand, is required for ribosomal stalk formation, an important event for the acquisition of functionality (Kemmler et al., 2009; Lo et al., 2009). The stalk recruits and activates elongation factors. It is composed of r-protein uL10 (yeast Rpp0), which anchors the stalk to the ribosome, and Rpp1 and Rpp2, which assemble in two heterodimers. Yvh1 removes the placeholder of uL10, Mrt4, from the cytoplasmic pre-60S subunit, thereby allowing the subsequent recruitment of translation factors.

Moreover, Nmd3, the essential NES containing transport adaptor, needs to be released and transported back to the nucleus. The GTPase Kre35 triggers the incorporation of the r-protein uL16 (yeast Rpl10), which leads to the displacement of Nmd3 (Hedges et al., 2005; West et al., 2005). After Arx1 release, uL16 incorporation (Bussiere et al., 2012) and stalk formation, the GTPase Efl1 triggers together with Sdo1 the release of Tif6 (Becam et al., 2001; Senger et al., 2001). Tif6 is a shuttling factor that binds to the joining face of the pre-60S subunit and prevents premature 40S subunit joining (Russell and Spremulli, 1979; Valenzuela et al., 1982). Using targeted

Figure 14. Cytoplasmic maturation of pre-60S subunits. Pre-60S subunits are exported into the cytoplasm bound to export factors (yellow) and shuttling trans-acting factors (green). The AAA-ATPase triggers the first release event in the cytoplasm, which consecutively leads to the sequential release of all other trans-acting factors. From Gerhardy et al., 2014.
proteomics, Altvater et al. discovered a plethora of shuttling pre-60S assembly factors that are released after Drg1-mediated release of Rlp24 (Altvater et al., 2012).

2.3. Preventing premature translation initiation

Adapted from Schütz and Panse 2012.

The complex structure of ribosomes presents a daunting challenge to cells, which need to monitor their correct assembly, transport and functionality (Strunk and Karbstein, 2009). Defects in the assembly of ribosomal subunits can compromise translational ability and fidelity, and they are often linked to disease (Freed et al., 2010). The precise decoding of genetic information by the ribosome is critical to all cellular processes. Therefore, conserved quality-control steps and checkpoints probably evolved simultaneously to ensure accuracy in ribosome production.

Cryo-EM image reconstructions previously revealed that several shuttling maturation factors occupy positions on late cytoplasmic 40S pre-ribosomes to prevent interactions with initiation factors, mRNAs and tRNAs (Strunk et al., 2011). Enp1 and Ltv1 block the mRNA channel opening by binding to uS3. Tsr1, Dim1 and Rio2 bind to the subunit interface of the pre-40S subunit, thereby preventing binding of initiation factor elF1A. elF3 binding is hindered by Pno1 and Nob1. These late cytoplasmic pre-40S particles mimic the translation initiation state of mature 40S subunits capable of interacting with mature 60S subunits to form an 80S-like particle (Figure 13) (Lebaron et al., 2012; Strunk et al., 2012). Strunk et al. propose that this translation-like interaction could provide a mechanism to test the translation potential of pre-40S subunits via their ability to bind 60S subunits and the translation initiation factor Fun12 (elF5B) before they are released into the translating pool (Figure 13).

What makes the 60S subunit competent to stimulate Nob1 activity? The maturation factor Tif6 prevents binding of pre-60S particles to mature 40S subunits. Tif6 is released from pre-60S subunits in the cytoplasm only after the formation of the acidic ribosomal stalk, and after the correct assembly of the
catalytic P-site (Bussiere et al., 2012; Panse and Johnson, 2010). Both studies suggest that only correctly assembled mature 60S subunits are competent to bind pre-40S particles and stimulate Nob1 activity (Lebaron et al., 2012; Strunk et al., 2012). Hence, the ability of 60S and pre-40S subunits to engage in translation initiation can be assessed simultaneously. The presence of both mature and immature ribosomal subunits in the cytoplasm suggests that strategies have evolved to assess their correct assembly, maturation state and functionality. Mature 60S and 40S subunits could be constantly interacting with each other and sensing their ‘decoding’ ability, potentially to segregate and target nonfunctional ribosomes for disassembly and degradation.

2.4. Chaperones in ribosome biogenesis

Many proteins need molecular chaperones to fold into their native structures. However, folding events involving one or a few polypeptides are relatively simple in comparison to the assembly of a ribosome. During ribosome biogenesis, transcription of rDNA, maturation and folding of rRNA need to be coordinated with translation of mRNAs encoding r-proteins, folding and accurately timed incorporation of all r-proteins into the maturing subunits. DExH/D box helicases help rRNAs to fold correctly (reviewed in Strunk and Karbstein, 2009), whereas small RNAs involved in ribosome biogenesis (e.g. the snoRNA snR30 (Fayet-Lebaron et al., 2009) or the snoRNA U3 (Hughes, 1996)) prevent premature contacts of rRNA fragments through base-pairing.

Ribosomal proteins are highly charged and often contain unstructured extensions that protrude into the ribosome RNA core (Figure 8 and Figure 9). Because they adopt extended conformations, individual ribosomal proteins are prone to aggregation, especially in presence of cytoplasmic polyanions, like RNA. Because of these features, ribosomal proteins are often not soluble when overexpressed in *Escherichia coli* (Culver and Noller, 2000). The mechanism know to prevent aggregation of ribosomal proteins are summarized here.

Molecular chaperones facilitate *de novo* protein folding (Frydman, 2001; Hartl and Hayer-Hartl, 2009; Kramer et al., 2009; McClellan et al., 2007),
assembly and disassembly of oligomeric complexes and quality control of misfolded and stress-denatured proteins (Bukau et al., 2006; McClellan et al., 2005). In eukaryotes, cytosolic chaperones can be divided into two groups, the stress inducible heat shock proteins and the chaperones linked to protein synthesis (CLIPS). Many ribosome associated chaperones bind to newly synthesized polypeptides (Albanese et al., 2006; Kramer et al., 2009; Wegrzyn and Deuerling, 2005). Ribosome associated chaperones include the nascent-polypeptide-associated complex (NAC) and the yeast Hsp70 homolog SSB (stress 70B), which function together with its cofactor RAC (ribosome-associated complex, Hsp40 in bacteria). Both chaperone systems dynamically bind the 60S subunit and interact with nascent polypeptides during biogenesis (Hundley et al., 2005; Pfund et al., 1998; Raue et al., 2007; Rospert et al., 2002; Wegrzyn and Deuerling, 2005). The Deuerling laboratory discovered that ribosomal proteins and ribosomal biogenesis factors aggregate upon SSB and NAC depletion (Koplin et al., 2010). This aggregation causes decreased levels of ribosomal subunits and functional ribosomes, suggesting that the SSB and NAC chaperone systems are essential for r-protein stabilization during and/or after translation. Other ribosome-associate chaperones do not directly bind to nascent chains. Neither RAC, composed of Zuo1 and Ssz1 (Gautschi et al., 2001), nor the RAC-like protein Jjj1 bind to newly synthesized polypeptides (Albanese et al., 2006; Yam et al., 2005). Jjj1 binds to Rei1 and thereby helps to recycle the 60S export factor Arx1 back into the nucleus (Demoinet et al., 2007; Meyer et al., 2007). The Frydman laboratory found a nuclear role for Zuo1 and Jjj1 in rRNA maturation (Albanese et al., 2010). It is speculated that Jjj1 and Zuo1 bind to specific locations within ribosomal precursors and help to either facilitate the recruitment and/or the dissociation of ribosomal proteins or assembly factors (Albanese et al., 2010).

In addition to their role as transport receptors, importins were proposed to have a dual role as cytoplasmic chaperones. Importins fulfill a stabilizing function on basic cargoes like histones and ribosomal proteins in the cytoplasm prior to nuclear import (Jäkel et al., 2002), shielding basic patches on the surface of their cargoes to prevent aggregation.
Recently, “private” chaperones for individual ribosomal proteins were identified. Yar1 has been suggested to bind to the r-protein uS3 (yeast Rps3) in the cytoplasm, accompany it into the nucleus and keep it soluble until its incorporation into pre-40S subunit (Koch et al., 2012). Two WD-repeat proteins, Rrb1 and Sqt1, are implicated in stabilization of ribosomal proteins and their targeting to ribosomal precursors. Rrb1 is required for early nucleolar maturation of the pre-ribosome and is thought to target uL3 (yeast Rpl3) to the 35S pre-rRNA (Iouk et al., 2001; Schaper et al., 2001). uL16 (yeast Rpl10) interacts with the essential protein Sqt1 (Eisinger et al., 1997), which is thought to facilitate its loading onto the pre-60S subunit. These findings suggest that more ribosomal proteins might depend on private chaperones for stabilization and targeting into pre-ribosomal particles.

2.5. Ribosomopathies

Deletion of ribosomal proteins is usually lethal. However, mutations in genes encoding either ribosomal proteins or factors involved in ribosome biogenesis can cause ribosomopathies, which are very rare disorders that vary greatly both in their physical manifestations and in their modes of inheritance. Despite the fact that ribosomes are essential in almost all cells, ribosomopathies do not affect all tissue and cell types and are usually tissue specific. Many ribosomopathies share similar phenotypes, including small stature, hematological defects, microcephaly, predisposition to cancer, and immune defects (Freed et al., 2010; Ganapathi and Shimamura, 2008; Khan et al., 2011; Luft, 2010). Identified ribosomopathies, mutated genes causing them, and characteristics linked to each type are summarized in Table 2.

2.5.1. Diamond-Blackfan anemia (DBA)

Diamond-Blackfan anemia belongs to the autosomal dominant forms of ribosomopathies. DBA is primarily characterized by a macrocytic anemia with reduced numbers of erythroid progenitors in the bone marrow and manifests usually in the first year of life (Choesmel et al., 2007; Draptchinskaia et al., 1999; Farrar and Dahl, 2011; Gazda et al., 2012; Idol et al., 2007).
<table>
<thead>
<tr>
<th>Disease</th>
<th>Clinical manifestations</th>
<th>Gene</th>
<th>Function in ribosome biogenesis</th>
<th>Occurrence</th>
<th>Putative mechanism of specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autosomal dominant</td>
<td>Macrocyclic anemia, bone marrow failure, craniofacial defects, cardiac defects, cancer predisposition, pre- and postnatal growth retardation, thumb abnormalities</td>
<td>RPS7, RPS10, RPS17, RPS19, RPS24, RPS26, RPS28, RPL5, RPL11, RPL26, RPL35A, TSR2</td>
<td>r-proteins, rRNA processing</td>
<td>1 in 100,000-1 in 200,000 live births</td>
<td>Translation of IRES-containing BAG1 and CSDE1 mRNAs in erythroid progenitors</td>
<td>(Choesmel et al., 2007; Cmejla et al., 2011; Doherty et al., 2010; Drapichinskaia et al., 1999; Gazda et al., 2012; Gripp et al., 2014; Horos et al., 2012; Idol et al., 2007)</td>
</tr>
<tr>
<td>Diamond-Blackfan anemia</td>
<td>Craniofacial abnormalities, mental retardation and psychomotor delay</td>
<td>TCOF1, POLR1D, POLR1C</td>
<td>Transcription of rRNA genes and proteins involved in rRNA processing</td>
<td>1 in 40,000-1 in 70,000</td>
<td>Treacle (encoded by TCOF1) strongly expressed in neural crest cells.</td>
<td>(Dauwverse et al., 2011; Dixon et al., 1997; Dixon et al., 2006; Gonzales et al., 2005; Sakai et al., 2012; Valduga et al., 2004)</td>
</tr>
<tr>
<td>Isolated congenital asplenia</td>
<td>Agenesis or hypoplasia of spleen leading to immunodeficiency</td>
<td>RPSA</td>
<td>40S r-protein</td>
<td>73 cases reported</td>
<td>Unknown</td>
<td>(Bolze et al., 2013; Gilbert et al., 2002; Mahlouji et al., 2011)</td>
</tr>
<tr>
<td>Aplasia cutis congenita</td>
<td>Agenesis of skin, usually on scalp vertex</td>
<td>BMS1</td>
<td>Ribosomal GTPase</td>
<td>&gt;500 cases</td>
<td>Unknown</td>
<td>(Gelperin et al., 2001; Mameros, 2013; Zhou et al., 2010)</td>
</tr>
<tr>
<td>Autosomal recessive</td>
<td>Pancreatic insufficiency, growth retardation, hematologic defects, skeletal abnormalities, cancer predisposition</td>
<td>SBDS</td>
<td>Release of eIF6 from pre-60S in allowing 40S subunit joining during cytoplasmic maturation.</td>
<td>1 in 76,000 live births</td>
<td>SBDS strongly expressed in developing pancreas</td>
<td>(Boocock et al., 2003; Finch et al., 2011; Ganapathi et al., 2007; Provost et al., 2012; Venkatasubramani and Mayer, 2008; Wong et al., 2011)</td>
</tr>
<tr>
<td>Shwachman-Diamond syndrome</td>
<td>Severe pre- and postnatal growth retardation, psychomotor retardation, microcephaly, micrognathia, joint contractures, rockerbottom feet</td>
<td>EMG1</td>
<td>Pseudouridine-N1-specific methyltransferase</td>
<td>1 in 355 live births in Hutterite population</td>
<td>Unknown</td>
<td>(Armistead et al., 2009; Lamont et al., 2005; Lowry et al., 2003; Meyer et al., 2011; Wurm et al., 2010)</td>
</tr>
<tr>
<td>Bowen-Conradi syndrome</td>
<td>Hypoplastic anemia, hypoplastic hair, short stature, immunological defects, hematological defects, malabsorption, cancer predisposition</td>
<td>RMRP</td>
<td>Pre-rRNA cleavage</td>
<td>Amish: 1 in 500-1 in 1,000, Finnish: 1 in 23,000</td>
<td>Short stature related to rRNA cleavage defect. Cancer predisposition putatively caused by defective cyclin B cleavage.</td>
<td>(Ridanpaa et al., 2001; Thiel et al., 2005; Thiel et al., 2007; Thiel and Rauch, 2011)</td>
</tr>
<tr>
<td>Year</td>
<td>Location</td>
<td>Case Reports</td>
<td>Pre-RNA Cleavage</td>
<td>Post-RNA Cleavage</td>
<td>Evidence for Pre-RNA Cleavage</td>
<td>Evidence for Post-RNA Cleavage</td>
</tr>
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</tr>
<tr>
<td>2006</td>
<td>Mexico, IL, USA</td>
<td>1 case reported</td>
<td>Yes</td>
<td>Yes</td>
<td>1 in 100,000</td>
<td>Large-scale microarray sequencing in 2006.</td>
</tr>
<tr>
<td>2010</td>
<td>North America, USA</td>
<td>2 cases reported</td>
<td>Yes</td>
<td>Yes</td>
<td>1 in 100,000</td>
<td>Large-scale microarray sequencing in 2006.</td>
</tr>
<tr>
<td>2011</td>
<td>Unknown</td>
<td>7 cases reported</td>
<td>Yes</td>
<td>Yes</td>
<td>1 in 100,000</td>
<td>Large-scale microarray sequencing in 2006.</td>
</tr>
</tbody>
</table>

- **Pre-RNA Cleavage**: Evidence for pre-RNA cleavage is reported in all cases.
- **Post-RNA Cleavage**: Evidence for post-RNA cleavage is reported in all cases.
- **Evidence**: Large-scale microarray sequencing was conducted in 2006.
DBA causes bone marrow failure, anemia, birth defects and increased risk of cancer (Greenspan et al., 1991; Lipton and Ellis, 2009). About 50% of DBA patients exhibit physical anomalies, including short stature and craniofacial abnormalities (Lipton and Ellis, 2009). In 60-70% of cases, mutations causing DBA are found in genes encoding r-proteins and result in the loss of function of these r-proteins. Reduced expression or haploinsufficiency of the r-protein results in ribosome biogenesis defects and causes reduced levels of 40S or 60S subunits.

Diamond-Blackfan anemia is caused by mutations in ribosomal proteins of the small subunit (eS19, eS7, eS10, eS17, eS24 and eS26) and ribosomal proteins of the large subunit (uL18, uL5, uL29, uL24). Recently, mutations in eS28 and in Tsr2, a direct binding partner of the r-protein eS26, were also reported to cause Diamond-Blackfan anemia with mandibulofacial dystosis (Treacher Collins syndrome) (Farrar and Dahl, 2011; Gripp et al., 2014; Willig et al., 1999). About 25% of all DBA cases are caused by mutations in eS19 (Draptchinskaia et al., 1999).

Although all DBA patients display defects in red blood cell maturation, the disorder is associated with various physical anomalies, which are challenging to correlate with a specific genotype (Gazda et al., 2008; Quarello et al., 2010). However, mutations in RPL5 correlate with craniofacial malformations, including cleft palate; and mutations in RPL5 and RPL11 cause thumb anomalies (Cmejla et al., 2009; Gazda et al., 2008). Moreover, a few DBA patients with mutations in RPS26 show rib and vertebrae malformations, associating DBA with the Klippel-Feil syndrome (KFS) (Cmejla et al., 2011). Klippel-Feil syndrome is characterized by a congenital fusion of two of the seven cervical vertebrae, causing neck shortness. It is speculated that mutations in eS26 might affect the translation of GDF6 and PAX1 (Cmejla et al., 2011), which regulate endochondral ossification and pattern formation during embryogenesis in vertebrates (McGaughran et al., 2003; Tassabehji et al., 2008). Rarely, patients have mutations in both RPS28 and TSR2, and show combined symptoms of DBA and Treacher Collins syndrome (TCS) (Gripp et al., 2014). TCS is a rare congenital autosomal dominant ribosomopathy characterized by craniofacial anomalies. TCS arises primarily
due to mutations in the TCOF1 gene, which encodes the nucleolar protein Treacle, which colocalizes with the upstream binding factor Ubf1 and RNA Pol I and participates in rDNA transcription (1996; Hayano et al., 2003; Valdez et al., 2004). During embryonic development, Treacle expression is specifically elevated in the neuroepithelium and the neural crest cell derived facial mesenchyme (Dixon et al., 2006).

It has been shown that, upon depletion of ribosomal proteins, the tumor suppressor p53 is activated in many DBA patients (Al-Rahawan et al., 2008; Dutt et al., 2011). Activation of p53 induces a checkpoint control ensuring the functionality and integrity of ribosomes (Boulon et al., 2010; Zhang and Lu, 2009) and appears to contribute to the pathogenesis in DBA. In non-stressed cells, levels of ribosomal proteins are balanced and p53 levels are low due to the ubiquitin ligase Hdm2/Mdm2, which induces constant ubiquitination and degradation of p53 (Figure 15A) (Haupt et al., 1997; Kubbutat et al., 1997). Phosphorylation of p53 and Hdm2/Mdm2 upon DNA damage triggers the complex dissociation and p53 activation (Chehab et al., 1999; Maya et al., 2001; Shieh et al., 1997). Activation of p53 results in the transcriptional upregulation of genes involved in cell cycle arrest, apoptosis and cellular senescence. Under ribosomal stress, many r-proteins move from the nucleolus to the nucleoplasm and are able to bind directly to Mdm2, thereby stabilizing and activating p53 (Figure 15B). When 40S biogenesis is impaired, p53 is activated in a uL5 (human Rpl11) dependent-manner: Cells selectively upregulate the translation of mRNAs containing 5' terminal oligopyrimidine tracts (5' TOP), including RPL11 (Fumagalli et al., 2009). Overexpressed uL5 enters the nucleus and interacts with Mdm2, thereby activating p53 (Figure 15C). uL18 (human Rpl5) and uL5 are thought to be the main regulators of the Hdm2/Mdm2 p53 pathway. In the nucleus, uL18 and uL5 form a trimeric complex with 5S rRNA, which, in its non-ribosome bound form, inhibits Hdm2 (Donati et al., 2013). Upon inhibition of ribosome biogenesis, r-proteins are normally synthesized but are degraded by ubiquitin-independent proteasomal degradation (Andersen et al., 2005; Lam et al., 2007; Tsvetkov et al., 2009; Warner, 1977). However, the uL18:uL5:5S rRNA complex, mutually protected from degradation (Bursac et al., 2012), binds to Mdm2 and triggers p53
activation (Figure 15D). Interestingly, eS26 can also trigger p53 activation through two different pathways. Reduced levels of eS26 cause ribosomal stress that activates p53 via uL5, whereas overexpression of eS26 activates p53 by direct binding to Mdm2 (Cui et al., 2014). These results highlight how balanced levels of r-proteins play important roles in health and disease.

**Figure 15. p53 regulation upon ribosomal stress.**
(A) Under non-stressed conditions, most r-proteins are imported into the nucleoplasm and are incorporated into pre-ribosomes in the nucleolus. Mdm2 constantly ubiquitinates and degrades p53. (B) Impairment in ribosome production causes ribosomal stress and mislocalizes r-proteins from the nucleolus to the nucleus. There, they directly interact with Mdm2 and as a consequence activate p53. (C) Impaired 40S biogenesis increases translation of the 5' TOP containing mRNA of RPL11. uL5 (encoded by RPL11) enters the nucleus and binds and inhibits Mdm2. (D) Upon inhibition of ribosome biogenesis, r-proteins are normally synthesized but are degraded by the proteasome to prevent the formation of toxic aggregates. The uL5:uL18:5S (L5-L11-5S) complex is protected from degradation and inhibits Mdm2. From Bursac et al., 2014.

Though the symptoms exhibited and r-proteins mutated in DBA are quite well characterized, it is still unclear why reduced levels of a r-protein affects cells in a tissue specific manner. One explanation is that different
cellular environments cause ribosomes to preferentially translate certain types of mRNAs. This was first discovered in virus-infected cells, where translation of viral mRNAs is favored over the translation of cap-containing cellular mRNAs (Jang et al., 1988; Nicholson et al., 1991). Viral mRNAs contain internal ribosome entry sites (IRESs) in their 5' UTR, which allow ribosome binding and translation of the respective mRNA. Moreover, IRESs are present in various cellular mRNAs. Mutations in eS19 and uL5 in mice result in deficient IRES-mediated translation of two genes, BAG1 and CSDE1, in erythroblasts (Horos et al., 2012). DBA patients also display reduced IRES-dependent translation of BAG1 and CSDE1 mRNAs, which are important for erythroid cell generation (Horos et al., 2012). Thus, ribosomal proteins can be involved in the recognition of specific mRNAs for translation (Armistead and Triggs-Raine, 2014). However, whether this is the case for all ribosomal proteins causing DBA, remains elusive.
AIM OF THE PROJECT

Within one generation time, a single yeast cell translates ~14 million ribosomal proteins that need to be efficiently targeted and incorporated into ribosomal precursors. After nuclear import, efficient targeting of the r-proteins to their assembly site in the nucleolus is crucial to build a functional eukaryotic ribosome. It is unclear how the import machinery interfaces with ribosome assembly and how intrinsically unstable and aggregation prone r-proteins are safely and efficiently targeted to emerging pre-ribosomes.

Like most r-proteins, eS26 contains large unstructured and basic regions that make it susceptible to aggregation and proteolysis. How eS26 is safely and efficiently targeted to the pre-ribosome remained unclear. Interestingly, mutations in eS26 are linked to the congenital disease Diamond-Blackfan anemia (DBA).

Using budding yeast as a model system and employing biochemical, cell-biological and structural approaches, we aimed to understand the mechanisms responsible for the efficient targeting to and incorporation of eS26 into the maturing pre-ribosome. Furthermore, we aimed at providing a mechanistic framework towards understanding eS26-associated DBA.
A RAN-GTP-INDEPENDENT MECHANISM ALLOWS RIBOSOMAL PROTEIN NUCLEAR IMPORT FOR RIBOSOME ASSEMBLY

From Schütz et al., 2014.

1. INTRODUCTION

Ribosome assembly is an essential process that is tightly connected to cellular growth and proliferation (Warner, 1999). In the eukaryotic model organism budding yeast, this universal translating machine is built of two subunits: a large subunit (60S) consisting of three different rRNAs (25S, 5.8S, 5S) and 46 ribosomal proteins (r-proteins) and a small subunit (40S) that contains a single rRNA (18S) and 33 r-proteins (Ben-Shem et al., 2011; Klinge et al., 2011; Rabl et al., 2011).

Assembly of the eukaryotic ribosome takes place in three distinct cellular territories: the nucleolus, the nucleoplasm and the cytoplasm (Gerhardy et al., 2014; Woolford and Baserga, 2013). RNA polymerase I drives production of the 35S pre-rRNA transcript in the nucleolus, which initiates the assembly process. The emerging 35S pre-rRNA transcript undergoes co-transcriptional modification and processing (Kos and Tollervey, 2010; Osheim et al., 2004), and associates primarily with 40S subunit r-proteins and ~50 assembly factors to form the earliest pre-ribosome, the 90S (Dragon et al., 2002; Grandi et al., 2002; Schäfer et al., 2003). Cleavage of 35S pre-rRNA releases the pre-40S particle, permitting the remaining pre-rRNA to associate with r-proteins of the 60S subunit and ~200 additional assembly factors to undergo further maturation and pre-rRNA processing (Fatica et al., 2002; Grandi et al., 2002; Nissan et al., 2002). Nuclear maturation of pre-ribosomal particles also requires the release of assembly factors, a process thought to require >50 energy-consuming enzymes (Kressler et al., 2010; Strunk and Karbstein, 2009). Export competent pre-ribosomal particles are separately transported through nuclear pore complexes (NPCs) into the cytoplasm by multiple export factors. In yeast, export factors include the exportin Crm1, which recognizes nuclear export sequences (NESs) in a Ran-GTP-dependent manner, and additional factors.
Export factors bind pre-ribosomal particles and interact simultaneously with FG-repeat nucleoporins lining the NPC channel (Altvater et al., 2012; Bassler et al., 2012; Bradatsch et al., 2007; Faza et al., 2012; Gadal et al., 2001; Hackmann et al., 2011; Johnson et al., 2001; Occhipinti et al., 2013; Oeffinger et al., 2004; Yao et al., 2008; Yao et al., 2010).

Following export, pre-ribosomal particles undergo final maturation prior to initiating translation. This involves the release of shuttling assembly factors, transport factors, incorporation of the remaining r-proteins and final pre-rRNA processing (Panse, 2011; Panse and Johnson, 2010). Within the pre-40S particle, immature 20S pre-rRNA is endonucleolytically cleaved into mature 18S rRNA by the nuclease Nob1 rendering the subunit translation competent (Fatica et al., 2004; Lamanna and Karbstein, 2009; Pertschy et al., 2009). Although, Nob1 is recruited to 40S pre-ribosomes in the nucleus, it is activated in the cytoplasm within an 80S-like pre-ribosomal particle formed upon interaction with a mature 60S subunit (Lebaron et al., 2012; Strunk et al., 2012). Additionally, multiple conserved ATPases Prp43, Rio2, Rli1 and Fap7, the Prp43-activator Pfa1, the kinase Rio1, the assembly factor Ltv1 and the r-protein uS11 (yeast Rps14) are implicated in this cleavage step (Geerlings et al., 2003; Granneman et al., 2005; Hellmich et al., 2013; Jakovljevic et al., 2004; Pertschy et al., 2009; Strunk et al., 2012; Vanrobays et al., 2003). Despite the identification of a plethora of factors and their general order of action, how nuclear and cytoplasmic assembly steps are coordinated remains largely unknown.

In addition to the tremendous energy required to assemble ribosomes, this process also accounts for the major proportion of the nucleocytoplasmic transport in a growing yeast cell (Rout et al., 1997; Sydorskyy et al., 2003). All mRNAs encoding r-proteins must be exported into the cytoplasm, where translation occurs. Nearly all newly synthesized r-proteins are then imported into the nucleus. In yeast, the importin Kap123 has been shown to be an important mediator of r-protein import, but the related importin Pse1 can functionally substitute Kap123 in vivo (Rout et al., 1997; Schlenstedt et al., 1997). Unlike other cargoes, r-proteins contain large unstructured regions that
form intricate interactions with rRNA within the mature ribosome and are prone to non-specific interactions with nucleic acids, aggregation and proteolytic degradation in their non-assembled state (Jäkel and Görlich, 1998; Jäkel et al., 2002; Klinge et al., 2011; Rabl et al., 2011). In contrast to typical protein transport events, nuclear import of r-proteins and subsequent transfer to the ribosome production site pose logistical challenges. In addition to their transport role, importins have been implicated to chaperone basic r-proteins during their transport to the nucleus (Jäkel et al., 2002). How these intrinsically unstable and aggregation-prone proteins are targeted to assembling pre-ribosomal particles after dissociating from importins remains unclear.

Here, we report the discovery of a carrier Tsr2 that coordinates transfer of the eukaryote specific r-protein eS26 (yeast Rps26; Ban et al., 2014) after nuclear import to the assembling 90S pre-ribosome. Tsr2 extracts eS26 from its importins to terminate its import process. Hereby, we reveal an atypical RanGTP-independent mechanism to dissociate an importin:cargo complex. Tsr2 binds and protects the released eS26 from aggregation and proteolysis thereby ensuring its safe transfer to the 90S pre-ribosome. Our data raise the possibility of a yet unidentified fleet of carriers that securely link the nuclear import machinery with the ribosome assembly pathway.

2. RESULTS

2.1. Tsr2 is required for cytoplasmic processing of 20S pre-rRNA to mature 18S rRNA

Previous genome-wide studies revealed a strong accumulation of immature 20S pre-rRNA in a TSR2 (Twenty S rRNA accumulation 2) deficient yeast strain (tsr2Δ)(Peng et al., 2003). Tsr2 is a conserved 23.7 kDa protein (Figure S1A) with no identified structural homologues that could provide clues into its role in 20S pre-rRNA processing. To dissect the function of Tsr2, we generated a conditional mutant in which the endogenous TSR2 was placed under the control of the GAL1 promoter (P<sub>GAL1</sub>-TSR2). On repressive glucose media, Tsr2 protein levels were undetectable and the P<sub>GAL1</sub>-TSR2 strain was
severely impaired in growth compared to a wild-type (WT) strain between 20-37°C (Figure 16A).

![Figure 16. Tsr2 predominantly localizes to the nucleus.](image)

(A) Tsr2-TAP, Tsr2-GFP and Tsr2-3xGFP cells are not impaired in growth. Left panel: Indicated strains were spotted on glucose containing media in 10-fold dilutions and grown at indicated temperatures for 3-7 days. Right panel: Tsr2 protein levels in whole cell extracts derived from the indicated strains were determined by Western analyses using α-Tsr2 antibodies. Protein levels of Arc1 served as loading control. (B) Tsr2 localizes predominantly to the nucleus. The Tsr2-TAP and the Tsr2-GFP strain and the \( P_{GAL1}^{-TSR2} \) strain containing a centrometric plasmids encoding Tsr2-3xGFP were grown at 30°C to mid-log phase. Localization of Tsr2-TAP was visualized by indirect immunofluorescence microscopy using polyclonal α-TAP antibody (red). Nuclear and mitochondrial DNA was stained with DAPI (blue). Localization of Tsr2-GFP and Tsr2-3xGFP was analyzed by fluorescence microscopy. Scale bar = 5 µm.
Next, we localized Tsr2 using an integrated C-terminal -GFP and -TAP tag at the genomic locus. These cell-biological studies revealed that both fusion proteins predominantly localize to the nucleus (Figure 16B). A similar location for the Tsr2-3xGFP fusion protein (expressed from a CEN plasmid under its natural promoter and terminator regions) was observed in a Tsr2-depleted strain. The strains expressing the various fusion proteins were not impaired in growth (Figure 16A) suggesting that addition of the -GFP, -TAP, and -3xGFP tags did not affect Tsr2 function. We conclude that Tsr2 mainly localizes to the nucleus.

The location of Tsr2 led us to test whether the accumulation of 20S pre-rRNA in tsr2Δ cells (Peng et al., 2003) is due to impaired nuclear export of pre-40S subunits. To this end, we monitored localization of 40S subunits in Tsr2-depleted cells using the established reporter uS5-GFP (yeast Rps2-GFP; Milkereit et al., 2001). We used the yrb2Δ mutant, which is specifically impaired in pre-40S subunit export, as a control (Moy and Silver, 2002). As expected, the yrb2Δ mutant showed a nuclear accumulation of uS5-GFP, in contrast to WT, which displayed cytoplasmic localization of this reporter (Figure S1B). Surprisingly, P_{GAL1}-TSR2 cells grown on glucose also showed cytoplasmic uS5-GFP localization (Figure S1B), indicating no apparent impairment in nuclear export of pre-40S subunits.

The data above raised the possibility that cytoplasmic processing of 20S pre-rRNA is impaired in Tsr2-depleted cells. To this end, we monitored the localization of the 5' portion of the internal transcribed spacer 1 (ITS1) that is present within immature 20S pre-rRNA, but not in mature 18S rRNA, by fluorescence in situ hybridization (FISH). In a WT strain, due to efficient nuclear export of pre-40S subunits, Cy3-ITS1 (red) is detectable only in the nucleolus (Figure 17A). After nuclear export, ITS1 is cleaved from 20S pre-rRNA by the endonuclease Nob1 and degraded by the exonuclease Xrn1 (Moy and Silver, 2002; Stevens et al., 1991). Tsr2-depleted cells exhibited strong cytoplasmic accumulation of Cy3-ITS1 (Figure 17A), indicating that cytoplasmic processing is impaired in these cells.
Figure 17. Tsr2 is required for cytoplasmic processing of 20S pre-rRNA to mature 18S rRNA.

(A) Tsr2-deficient cells accumulate immature 20S pre-rRNA in the cytoplasm. WT and \( P_{\text{GAL1-TSR2}} \) cells were grown at 30°C in glucose containing media to mid-log phase. Localization of 20S pre-rRNA was analyzed by FISH using a Cy3-labeled oligonucleotide complementary to the 5' portion of ITS1 (red). Nuclear and mitochondrial DNA was stained with DAPI (blue). Scale bar = 5 \( \mu \)m. (B) Tsr2-depleted cells accumulate 80S-like particles. WT and \( P_{\text{GAL1-TSR2}} \) cells were grown at 30°C in glucose containing media to mid-log phase. Cell extracts were prepared after cycloheximde treatment to preserve polysomes and subjected to sedimentation centrifugation on 7-50% sucrose gradients. Polysome profiles at OD\textsubscript{254nm} were recorded and the peaks for 40S and 60S subunits, 80S ribosomes and polysomes are indicated (top panels). The gradients were fractionated and the RNA was extracted, separated on a 2% Agarose gel, stained with GelRed (Biotium, middle panels) and subsequently analyzed by Northern Blotting using probes against indicated rRNAs (bottom panels). Exposure times for phosphoimager screens were 20 min for 25S and 18S rRNA, and 3-4 h for 20S pre-rRNAs.
Two studies proposed that 20S pre-rRNA processing occurs within an 80S-like particle formed \textit{via} interaction between a mature 60S subunit and a pre-40S subunit in the cytoplasm (Lebaron \textit{et al.}, 2012; Strunk \textit{et al.}, 2012). One possibility is that formation of this particle is impaired in Tsr2-depleted cells, thereby indirectly interfering with 20S pre-rRNA processing. To test this, we performed polysome analyses. Cell extracts from WT and Tsr2-depleted cells prepared under polysome preserving conditions were analyzed by sucrose gradient centrifugation. In agreement with a role in the 40S biogenesis pathway, the polysome profile of Tsr2-depleted cell extracts revealed strongly reduced levels of free 40S subunits and polysomes (Figure 17B, top panel). Northern analyses revealed that mature 25S rRNA and immature 20S pre-rRNA co-peak (Figure 17B, bottom panel), indicating accumulation of 80S-like particles, similar to the one seen upon Fap7-depletion (Granneman \textit{et al.}, 2005; Strunk \textit{et al.}, 2012). Thus, pre-40S subunits that are exported into the cytoplasm in Tsr2-depleted cells interact with mature 60S subunits, but fail to undergo 20S pre-rRNA processing. We conclude that Tsr2 is required for cytoplasmic maturation of pre-40S subunits.

2.2. \textbf{Tsr2 directly binds the eukaryotic-specific r-protein eS26}

Next, we analyzed the sedimentation behavior of Tsr2 on sucrose density gradients. Cell extracts from WT cells were subjected to polysome analyses. The gradient was fractionated and analyzed by Western analyses. Unexpectedly, Tsr2 did not co-sediment with the 40S peak or with heavier fractions, but was found exclusively in lighter fractions at the top of the gradient (Figure 18A). These data indicate that Tsr2 does not stably associate with pre-ribosomal particles in the 40S biogenesis pathway.

To identify interaction partners of Tsr2, we isolated Tsr2-TAP. In agreement with the sedimentation studies above, Tsr2-TAP did not isolate a pre-40S particle. Instead, Tsr2-TAP co-enriched stoichiometric amounts of the eukaryotic-specific r-protein eS26 (Figure 18B; Peng \textit{et al.}, 2003). Further, yeast two-hybrid analysis revealed a strong interaction between Tsr2 and eS26, as determined by growth on stringent adenine deficient media (Figure
In vitro binding studies using recombinant proteins showed that eS26 and Tsr2 formed a robust complex (Figure 18D). We conclude that eS26 directly binds Tsr2.

**Figure 18. Tsr2 directly binds eS26.**

(A) Tsr2 does not co-sediment with 40S subunits. WT cells were grown at 30°C to mid-log phase, extracts were prepared and fractionated as described in Figure 17B. The polysome profile at OD_{254nm} is shown in the upper panel. The peaks for 40S and 60S subunits, 80S ribosomes and polysomes are indicated. The gradient was fractionated, TCA precipitated and the protein content was assessed by Western analyses using the indicated antibodies. (B) Tsr2-TAP co-enriches the r-protein eS26. Tsr2-TAP was isolated by tandem affinity purification and the Calmodulin-eluate was separated by 4-12% gradient SDS-PAGE and analyzed by Silver staining. The indicated proteins were identified by mass spectrometry. (C) Tsr2 interacts with eS26 in a yeast two-hybrid assay. Plasmids encoding the indicated GAL4 DNA-binding domain (BD) and GAL4 activation domain (AD) fusion proteins were transformed into the yeast reporter strain NMY32. Transformants were spotted in 10-fold serial dilutions onto SDC-Leu-Trp (-Leu-Trp) or SDC-Ade (-Ade) and incubated at 30°C for 4 days. Growth on SDC-Ade indicates a strong two-hybrid interaction. The SV40 Large T antigen served as negative control for these analyses. (D) Tsr2 directly binds eS26 in vitro. GST-Tsr2 was immobilized on Glutathione Sepharose before incubation with an E. coli lysate containing recombinant eS26. After incubation, bound proteins were eluted by SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining. L= input.
2.3. eS26 is required for cytoplasmic processing of 20S pre-rRNA

In budding yeast, two non-essential genes, RPS26A and RPS26B, encode the r-protein eS26. To investigate the phenotypes of RPS26 deficiency, we created a conditional double mutant in which the endogenous promoter of RPS26A in the rps26bΔ strain was replaced with the GAL1 promoter (P_{GAL1}-RPS26A). Consistent with an essential function of eS26 in yeast, the P_{GAL1}-RPS26Arps26bΔ strain did not grow on repressive glucose containing medium (Figure 19A). Using this strain, we investigated whether eS26 is required for nuclear export of pre-40S subunits and/or cytoplasmic 20S pre-rRNA processing by monitoring the localization of uS5-GFP and Cy3-ITS1. eS26-depletion did not induce nuclear accumulation of uS5-GFP (Figure S1B), indicating no apparent impairment in pre-40S subunit nuclear export. However, these cells showed a strong cytoplasmic accumulation of Cy3-ITS1 (Figure 19B), indicating impairment in final 20S pre-rRNA processing. Further, polysome analyses of eS26-depleted cell extracts revealed strongly reduced levels of free 40S subunits (Figure 19C, top panel). Northern analyses revealed that mature 25S rRNA and immature 20S pre-rRNA co-peaked (Figure 19C, bottom panel), indicating an accumulation of 80S-like particles. Thus, as observed in Tsr2-depleted cells, eS26-depleted cells contain pre-40S subunits that fail to process 20S pre-rRNA in the cytoplasm. Based on these data we conclude that eS26 is required for cytoplasmic maturation of pre-40S subunits.

2.4. eS26 is recruited to the earliest pre-ribosomal particle, the 90S

The robust interaction between the predominantly nuclear localized Tsr2 and eS26 prompted us to investigate at which stage eS26 is recruited to pre-40S subunits. To address this, we isolated pre-ribosomal particles at different maturation stages along the 40S biogenesis pathway (Grandi et al., 2002; Schäfer et al., 2003). Noc4-TAP purifies the earliest precursor of the pre-40S subunit, the 90S pre-ribosome; Enp1-TAP purifies both the 90S and early pre-40S subunits; Rio2-TAP purifies a late pre-40S subunit containing
Figure 19. eS26 is required for cytoplasmic processing of immature 20S pre-rRNA to mature 18S rRNA.
(A) eS26 is essential for viability in yeast. Left panel: WT, rps26aΔ, rps26bΔ and the conditional mutant P\_GAL1-RPS26Arps26bΔ were spotted in 10-fold dilutions on galactose and repressive glucose containing media and grown at 30°C for 2-4 days. Right panel: Protein levels of eS26 in whole cell extracts of indicated strains were determined by Western analyses using α-eS26 antibodies. Arc1 protein levels served as loading control. (B) eS26-depleted cells accumulate immature 20S pre-rRNA in the cytoplasm. P\_GAL1-RPS26Arps26bΔ cells transformed with indicated plasmids were grown in glucose containing liquid media at 37°C to mid-log phase. Localization of 20S pre-rRNA was analyzed by FISH using a Cy3-labeled oligonucleotide complementary to the 5′ portion of ITS1 (red). Nuclear and mitochondrial DNA was stained with DAPI (blue). Scale bar = 5 µm. (C) eS26-depleted cells accumulate 80S-like particles. The indicated strains were grown in glucose containing liquid media at 30°C to mid-log phase. Cell extracts were prepared after cycloheximide treatment and subjected to sedimentation centrifugation on 7-50% sucrose density gradients. Polysome profiles were recorded at OD\_254nm (top panels). The peaks for 40S and 60S subunits, 80S ribosomes and polysomes are indicated. Sucrose gradients were fractionated, the RNA was extracted, separated on a 2% Agarose gel, stained with GelRed (Biotium, middle panels) and subsequently analyzed by Northern blotting using probes against the indicated rRNAs (bottom panels). Exposure times for phosphoimager screens were 20 min for 25S and 18S rRNA, and 3-4 h for 20S pre-rRNAs.

immature 20S pre-rRNA; and Asc1-TAP purifies a 40S subunit containing mature 18S rRNA and devoid of late assembly factors (Figure 20A and Figure S2A). Co-enrichment of eS26 with pre-ribosomal particles was assessed by (1) Western analyses using antibodies that recognize eS26 and (2) selected reaction monitoring mass spectrometry (SRM-MS). SRM-MS is a reliable tool that overcomes stochastic under sampling of peptides, a critical deficit in shotgun mass spectrometry which complicates the reproducible precise quantitation of proteins of interest present in a complex mixture (Picotti and Aebersold, 2012). SRM relies on the development of specific mass spectrometric-based assays for every target protein and their subsequent application to the relative or absolute quantification within multiple biological samples. We developed a set of SRM assays that enabled us to simultaneously monitor the co-enrichment of eS26 and different r-proteins: uS7 (Rps5), eS28 (Rps28), eS1 (Rps1) and uS11 (Rps14) (Figure 20B) with multiple pre-ribosomal particles. Both Western and SRM analyses revealed that eS26 co-enriches efficiently with the earliest ribosomal precursor, the 90S, and different pre-ribosomes along the 40S maturation pathway (Figure 20A and Figure 20B). The Western signal for eS26 on the 90S pre-ribosome is specific since no association was detected with the earliest 60S pre-ribosome (Ssf1-TAP) (Figure S2B).
Figure 20. eS26 is incorporated into the earliest pre-ribosome, the 90S.

(A) eS26 co-enriches with pre-ribosomal particles along the 40S maturation pathway. Pre-ribosomal particles in the 40S maturation pathway were purified using the indicated TAP-tagged baits. Calmodulin-eluates were analyzed by Silver staining and Western analyses using the indicated antibodies. The r-protein uS7 served as loading controls for the TAPs. (B) SRM-MS reveals co-enrichment of eS26 with pre-ribosomal particles. Upper panel: The relative abundance of different r-proteins was normalized to uS7 levels in the indicated TAP purifications (three independent biological replicates). The error bars show the standard deviation. Lower panel: The intensity of different transitions (listed in the box) of two specific peptides of eS26 was determined by SRM mass spectrometry in the indicated TAP purifications. (C) eS26-GFP accumulates in the nucleus in a yrb2Δ strain. Left panel: WT, rps26aΔ, and RPS26A-GFP cells were spotted in 10-fold dilutions and grown at indicated temperatures for 3-7 days. Right panel: WT and yrb2Δ cells expressing eS26-GFP were grown in glucose containing liquid media to mid-log phase at 20°C. Localization of eS26-GFP was monitored by fluorescence microscopy. Scale bar = 5 µm.
To support these biochemical data, we performed a complementary cell-biological experiment. If eS26 were targeted to the 90S pre-ribosome, then impairment in pre-40S subunit export should result in its accumulation in the nucleus. To monitor eS26 localization in vivo, we tagged RPS26A with GFP at the C-terminus (eS26-GFP) in WT and yrb2Δ cells at the genomic locus. Unlike the rps26aΔ mutant, the RPS26A-GFP strain was not impaired in growth at 20°C and 37°C indicating that the addition of GFP does not impair its function on the 40S subunit (Figure 20C, left panel). As expected, WT cells displayed a strong cytoplasmic localization of eS26-GFP (Figure 20C, right panel). In contrast, the yrb2Δ mutant accumulated eS26-GFP in the nucleus (Figure 20C, right panel). Together all these data suggest that eS26 is transported to the nucleus for loading on the 90S pre-ribosome.

Consistent with the sedimentation studies and direct binding to only eS26 (Figure 18), Tsr2 did not detectably co-enrich with affinity purified pre-ribosomal particles in the 40S maturation pathway (Figure 20A). This lack of co-enrichment was not due to altered protein levels in the different TAP strains, since Western analyses of whole cell extracts revealed that Tsr2 was expressed at WT levels (Figure S2C). Altogether, these results suggest that there are at least two populations of eS26 in vivo, one bound to ribosomes and another bound to Tsr2.

2.5. eS26 is imported primarily by Kap123 and Kap104 into the nucleus

We next investigated how eS26 is imported into the nucleus prior to its incorporation into the 90S pre-ribosome. In yeast, the most abundant importin Kap123 transports various r-proteins into the nucleus (Rout et al., 1997; Schlenstedt et al., 1997). However, r-proteins also utilize additional importins, including Pse1, Kap104, Sxm1 and Nmd5 (Rout et al., 1997; Sydorskyy et al., 2003). We investigated the interaction between eS26 and all yeast importins in vitro. These studies revealed that the importins Kap123, Kap104 and Pse1 efficiently bound eS26 (Figure 21A). A very weak interaction was observed between Sxm1, Kap95 and Nmd5 and eS26, and no binding was observed
with the remaining importins (Figure S3). In contrast, none of the importins bound to either Tsr2 or the Tsr2:eS26 complex (Figure 21A and Figure S3), indicating that eS26 alone specifically interacts with importins.

To verify our interaction data in vivo, we monitored nuclear uptake of eS26 in WT cells and in different importin mutants. The r-protein eS26 is assembled into the 90S pre-ribosome and is then rapidly transported to the cytoplasm as part of the 40S pre-ribosome. To investigate eS26 nuclear uptake in vivo we uncoupled its import from its export. Structural analyses of the 40S subunit showed that the N-terminus of eS26 is embedded within the rRNA framework (Figure S4A; Rabl et al., 2011). We fused GFP to the N-terminus of eS26 with the aim to impair its incorporation into the 90S pre-ribosome. Sucrose gradient analyses showed that GFP-eS26 co-sediments only in lighter fractions at the top of the gradient suggesting that it is not incorporated into pre-ribosomes (Figure S4B). In vitro binding studies showed that like eS26, GFP-eS26 interacts with Kap123, Kap104 and Pse1 (Figure S4C). Thus, the GFP-eS26 fusion protein is functional to recruit the import machinery, although it does not complement the eS26-depleted strain (Figure S5A). Further, GFP-eS26 directly binds Tsr2 (Figure S4C) and importantly, like eS26, GFP-eS26 is degraded upon Tsr2-depletion (Figure S5B). We exploited the GFP-eS26 fusion protein as a tool to monitor the nuclear uptake of eS26 in different importin mutants. Consistent with in vitro binding assays, nuclear uptake of GFP-eS26 was reduced in kap123Δ and kap104Δ cells (Figure 21B), indicating that eS26 import requires these importins. Nuclear localization of GFP-eS26 in the pse1-1 ts mutant at restrictive temperature remained unaffected (Figure 21B) indicating that impairment of this importin alone does not inhibit the nuclear import of eS26. The pse1-1 kap123Δ mutant showed only a slight increase in cytoplasmic staining of GFP-eS26 Figure 21B). Nuclear import of GFP-eS26 was unaffected in the kap114Δ sxm1Δ double mutant and sxm1Δ kap120Δ nmd5Δ triple mutant (Figure 21B).

Next, we investigated which region of eS26 contributes to its nuclear uptake. For this, we monitored the localization of different truncated versions of eS26 fused to -GFP at the N-terminus. These cell-biological analyses revealed that the Zn$^{2+}$-binding domain is required for efficient nuclear uptake
Figure 21. Kap123, Kap104 and Pse1 transport eS26 to the nucleus.

(A) eS26, but not Tsr2:eS26 or Tsr2, interacts with Kap123, Kap104 and Pse1. Recombinant, GST-Kap123, GST-Kap104, GST-Pse1 and GST alone were immobilized on Glutathione Sepharose and incubated with purified 3.4 µM Tsr2, 4 µM Tsr2:eS26, or E. coli lysate containing ~4 µM eS26 in PBSKMT combined with competing E. coli lysates for 1 h at 4°C. After washing with PBSKMT, bound proteins were eluted in SDS sample buffer and separated by SDS-PAGE. Proteins were visualized by Coomassie Blue staining or Western analyses using indicated antibodies. L= input. GST-tagged importins are indicated with asterisks.

(B) Nuclear uptake of GFP-eS26 is impaired in kap123∆ and kap104∆ mutants. Strains expressing GFP-eS26 were grown in synthetic media at 25°C (ts-mutants: pse1-1 and kap104∆) or 30°C to mid-log phase. Ts-mutant strains were then shifted to 37°C for 4 h and localization of GFP-eS26 was analyzed by fluorescence microscopy. Percentage of cells displaying cytoplasmic mislocalization of the GFP-eS26 fusion is indicated. Scale bar = 5 µm.

(C) Tsr2-3xGFP is targeted to the nucleus by Kap123. Importin mutant strains expressing Tsr2-3xGFP were grown in synthetic media at 25°C (ts-mutants: pse1-1 and kap104∆) or 30°C to mid-log phase. Pse1-1 and kap104∆ cells were then shifted to 37°C for 4 h. P_GAL1-RPS26Arps26b∆ cells containing Tsr2-3xGFP were grown for 15 h in glucose containing media. Localization of Tsr2-3xGFP was analyzed by fluorescence microscopy. Scale bar = 5 µm.
of eS26 (Figure S5C). If eS26 were imported into the nucleus in complex with Tsr2, then we reasoned that depletion of eS26 would induce Tsr2 mislocalization to the cytoplasm. However, localization of Tsr2-3xGFP was not affected upon eS26-depletion (Figure 21C). These cell-biological studies together with the observation that Tsr2:eS26 complex is unable to recruit importins argue against the idea that eS26 is transported to the nucleus in complex with Tsr2. We conclude that Kap123 and Kap104 target eS26 to the nucleus and that Tsr2 is not a component of this import complex.

2.6. Kap123 targets Tsr2 to the nucleus

Next, we investigated how Tsr2 is targeted to the nucleus in vivo. For this, we monitored the location of Tsr2-3xGFP in different importin mutants. We found that Tsr2-3xGFP mislocalizes to the cytoplasm in the kap123Δ mutant, but not in other importin mutants for e.g. kap104Δ and pse1-1 (Figure 21C). Thus, Kap123 seems to be the major import receptor for Tsr2. However, we did not observe a direct interaction between Tsr2 and Kap123 or any other importin in vitro (Figure 21A and Figure S3). One possibility could be that import of Tsr2 by Kap123 is regulated by post-translational modification. Alternatively, Tsr2 might be transported into the nucleus via a “piggy bag” mechanism bound to another yet unknown Kap123 cargo. We can exclude the possibility that eS26 serves as an adaptor to import Tsr2 since (1) Tsr2 does not mislocalize to the cytoplasm in a eS26-depleted strain (Figure 21C) and (2) in vitro binding assays show that the Tsr2:eS26 complex does not interact with Kap123 (Figure 21A).

2.7. Tsr2 dissociates importin:eS26 complexes in a RanGTP-independent manner

After transport of an importin:cargo complex into the nucleus, RanGTP binds to the N-terminal region of the importin, triggering cargo release and allowing recycling of the importin to participate in subsequent import cycles (Cook et al., 2007; Kobayashi and Matsuura, 2013; Lee et al., 2005). To test
Figure 22. Tsr2 efficiently dissociates eS26 from importins in a RanGTP-independent manner.

(A) RanGTP (His$_6$-Gsp1Q71L-GTP) does not efficiently release eS26 from Kap123 and Pse1. GST-importin:eS26$_{\text{FLAG}}$ complexes immobilized on Glutathione Sepharose were incubated with either buffer alone or with 1.5 µM RanGTP or 3 nM 3' end of 18S rRNA for 1 h at 4°C. Washing, elution, and visualization were performed as in Figure 21A. GST-tagged importins are indicated with asterisks. (B) Tsr2 efficiently dissociates the Kap123:eS26$_{\text{FLAG}}$ complex. The GST-Kap123: eS26$_{\text{FLAG}}$ complex immobilized on Glutathione Sepharose was incubated with either buffer alone or with 1.5 µM or 375 nM RanGTP or 1.5 µM or 375 nM Tsr2. Samples were withdrawn at the indicated time points. Washing, elution, and visualization were performed as in Figure 21A. GST-tagged Kap123 is indicated with an asterisk.
whether the release of eS26 from the importins is RanGTP-dependent, we performed in vitro dissociation assays. A pre-formed importin:eS26 complex was incubated with 1.5µM Gsp1Q71L-GTP (equivalent to the human RanQ69L mutant that cannot efficiently hydrolyze GTP, hereafter Gsp1Q71L-GTP is termed RanGTP; Bischoff et al., 1994; Maurer et al., 2001). Although RanGTP was able to dissociate the Kap104:eS26 complex, and partially dissociate the Pse1:eS26 complex, we did not observe dissociation of the Kap123:eS26 complex even after 1 h incubation (Figure 22A). It was reported that both RNA and RanGTP are required to release of the mRNA binding proteins Nab2 and Nab4 from Kap104 and the mRNA export factor Npl3 from Mtr10 (Lee and Aitchison, 1999; Senger et al., 1998). Because eS26 directly interacts with the 3’ end of the 18S rRNA (Figure S6, lower panel), we tested if this region of the 18S rRNA is required to release eS26 from Kap123. However, eS26 remained stably bound to Kap123 in the presence of this RNA, either alone or in combination with RanGTP (Figure 22A and Figure S7A).

Since the Tsr2:eS26 complex was unable to interact with importins, we tested whether Tsr2 stimulates the release of eS26 from importins. Surprisingly, Tsr2 alone efficiently removed eS26 from Kap123, Pse1 and Kap104 (Figure S7A and Figure 23 and data not shown). This release was specific, since only RanGTP, but not Tsr2, was able to remove the 40S assembly factor Slx9 (Faza et al., 2012) from the Pse1:Slx9 complex under the same conditions (Figure S7B). Moreover, Tsr2 specifically releases eS26 from the importin:eS26 complex, since it did not dissociate other tested importin:r-protein complexes (Kap123:uS14, Kap123:eS31 and Kap123:eS8) (Figure S8).

Since eS26 was inefficiently removed from the Pse1:eS26 complex after 1 h incubation with RanGTP (Figure 22A), we investigated the dissociation kinetics of importin:eS26 complexes in the presence of RanGTP or Tsr2. For this, the importin:eS26 complex was incubated with 1.5 µM of either RanGTP or Tsr2 and the release of eS26 from the importin was monitored over time. We found that the amount of eS26 bound to Kap123, Pse1 or Kap104 was only slightly reduced after 8 min, even though RanGTP
was efficiently recruited to the different importin:eS26 complexes (Figure 22B, left panel and Figure S9, left panel). In contrast, Tsr2 completely removed eS26 from these importins within 1 min incubation (Figure 22B, right panel and Figure S9, right panel). Notably, even at lower concentrations (375 nM) Tsr2 was able to release eS26 from the importin:eS26 complex (Figure 22B and Figure S9). Moreover, Tsr2 stably associated with the released eS26 (Figure 23). These data show that Tsr2 efficiently removes eS26 from importins in the absence of RanGTP, and that Tsr2 remains stably bound to eS26.

Figure 23. eS26 stably associates with Tsr2 after its release from Kap123. Left panel indicates the experimental setup as flowchart. Immobilized GST-Kap123:eS26FLAG and GST-Pse1:eS26FLAG complexes were incubated with 1.5 µM His6-Tsr2 or buffer alone. As shown in the flowchart, the supernatant was incubated with Ni-NTA Agarose for 1 h at 4°C (IP-Sup). Washing, elution, and visualization were performed as in Figure 21A. GST-tagged Kap123 and Pse1 are indicated with asterisks.
2.8. Tsr2 shields eS26 from proteolysis and aggregation, and promotes a safe transfer to the 90S pre-ribosome

The observation that Tsr2 is able to extract eS26 from importins, prompted us to investigate whether Tsr2 plays a role in the transfer of eS26 to the assembling pre-ribosome. To test this, we isolated Enp1-TAP, which purifies both the 90S pre-ribosome and an early pre-40S subunit, from WT and Tsr2-depleted cells and assessed co-enrichment of eS26 by Western analyses. Consistent with a role for Tsr2 in supplying eS26 to the 90S pre-ribosome, we found that eS26 does not efficiently co-enrich with Enp1-TAP in Tsr2-depleted cells (Figure 24A). This was specific for eS26, since the recruitment of uS7 and uS3 to Enp1-TAP particles was not affected in these cells (Figure 24A). This lack of enrichment was due to decreased eS26 protein levels, since Western analyses of whole cell extracts derived from Tsr2-depleted cells revealed strongly reduced eS26 protein levels (Figure 24B). These data led us to test whether eS26 becomes susceptible to proteolysis in Tsr2-depleted cells. To this end, we monitored eS26 protein levels over time in whole cell extracts after switching the P<sub>GAL1-TSR2</sub> strain to repressive glucose containing media. These analyses revealed that eS26 protein levels decreased over time upon Tsr2-depletion (Figure 24C). We observed that purified recombinant eS26 was highly prone to aggregation. Expressing eS26 as a fusion protein with a highly soluble GST tag suppressed its tendency to aggregate. However, removal of the GST tag after cleavage by PreScission protease resulted in immediate aggregation of free eS26, as determined by a massive increase in the light scattering intensity (Figure 24D). We tested whether Tsr2 could suppress the aggregating ability of recombinant eS26. We treated GST-eS26 with PreScission protease in absence and presence of Tsr2. A concomitant decrease in the light scattering of the reaction mixture was observed (Figure 24D), as the Tsr2 concentration in the cleavage buffer was increased, indicating aggregation of free eS26 was suppressed. Altogether, these data indicate that Tsr2 protects eS26, thereby ensuring a safe transfer to the 90S pre-ribosome.
Figure 24. Tsr2 shields eS26 from proteolysis and aggregation, and promotes safe transfer to the 90S pre-ribosome.

(A) Efficient recruitment of eS26 to Enp1-TAP requires Tsr2. Enp1-TAP was isolated from WT and Tsr2-depleted cells. After tandem affinity purification, eluates were separated by 4–12% gradient SDS-PAGE and subjected to Western analyses using indicated antibodies. CBP (α-TAP) levels served as loading control. (B) eS26 levels are strongly reduced in Tsr2-depleted cells. Whole cell extracts (WCE) prepared from WT and Tsr2-depleted cells were assessed by Western analyses using antibodies against the indicated proteins. Arc1 protein levels served as loading control. (C) Tsr2 protects eS26 from proteolysis in vivo. The conditional mutant strain P_{GAL1}^{+}TSR2 growing on galactose medium was transferred to repressive glucose containing liquid media at 30°C. Cells were withdrawn at the indicated time points and whole cell extracts were prepared. Western analyses were performed to determine the levels of the indicated proteins. Arc1 served as loading control. (D) Tsr2 prevents aggregation of recombinant eS26 in vitro. The aggregation assay was performed in a 384-well plate. In each well 33 µM GST-eS26 and a given concentration of Tsr2 (0 up to 266 µM) in PBSKMT was pre-incubated for 1 h at 4°C (final volume: 90 µl). 250 nM of PreScission protease was added to initiate aggregation. After 1 h of incubation, the scattering signal of the aggregated eS26 was monitored by a 384-well plate reader by measuring the intensity at 450 nm (Y-axes). Concentration of Tsr2 used in the assay (X-axes) are expressed as a molar ratio of eS26:Tsr2. Four replicates for each well were measured. The error bars show the standard deviation.
2.9. An eS26 mutant associated with Klippel-Feil syndrome in Diamond-Blackfan anemia patients is impaired in importin binding

Mutations in r-proteins have been linked to Diamond-Blackfan anemia (DBA), a rare congenital red blood cell aplasia (Ellis, 2014; Ellis and Gleizes, 2011; Ellis and Lipton, 2008; Ganapathi and Shimamura, 2008; McCann and Baserga, 2013; Narla and Ebert, 2010). Several mutations in the start codon of RPS26, including two mutations within eS26, D33N and C77W have been linked to DBA (Cmejla et al., 2011; Doherty et al., 2010). Both residues are highly conserved from yeast to humans (Figure S6, upper panel). The C77W mutation is additionally linked to Klippel-Feil syndrome (KFS), a skeletal developmental disorder in DBA patients (Cmejla et al., 2011).

In order to analyze the phenotypes induced by the D33N and C77W mutations, we introduced the individual mutations into yeast RPS26A. First, plasmids encoding DBA-linked mutants were transformed into the P\textsubscript{GAL1}-RPS26Arps26b\textDelta strain and growth was analyzed on glucose containing media. Whereas the D33N mutant partially rescued the lethality of the eS26-conditional mutant, the C77W variant did not allow any growth (Figure 25A). Further, as in the P\textsubscript{GAL1}-TSR2 strain under repressive conditions, both variants resulted in strongly reduced eS26 protein levels (Figure 25B). Neither strain displayed defects in the nuclear export of pre-40S subunits (Figure S1B). As expected, neither variant was able to rescue the 20S pre-rRNA processing defect of eS26-deficient cells, as determined by the strong cytoplasmic localization of Cy3-ITS1 (Figure 25C). Thus, eS26 mutants linked to DBA are impaired in cytoplasmic processing of 20S pre-rRNA.

We tested whether the identified eS26 binders could interact with D33N and C77W variants in vitro. Pull-down assays demonstrated that both mutant proteins efficiently bound Tsr2 (Figure 26A), suggesting these mutations do not contribute to the Tsr2:eS26 interaction surface. The eS26D33N mutant efficiently binds to Kap123, Kap104 and Pse1 (Figure 26B). In agreement with these interaction studies, nuclear uptake of GFP-eS26D33N was not affected (Figure 26C) and the levels of GFP-eS26D33N were strongly reduced upon Tsr2-depletion (Figure S5B). In contrast, the eS26C77W mutant interacted
Figure 25. The DBA and KFS mutants are impaired in cytoplasmic 20S pre-rRNA processing.

(A) The DBA linked eS26D33N and eS26C77W mutants are unable to fully rescue the growth defect of eS26-depleted cells. The $P_{GAL1}$-RPS26Arps26bΔ strain transformed with different plasmids encoding eS26 mutants were spotted in 10-fold dilutions on selective glucose containing plates and grown at indicated temperatures for 3-7 days. Residues mutated in DBA are depicted in Figure S6. (B) DBA linked mutations cause strongly reduced eS26 protein levels. Whole cell extracts were prepared from $P_{GAL1}$-RPS26Arps26bΔ cells transformed with indicated plasmids encoding for eS26 WT and mutant proteins. eS26 protein levels were assessed by Western analyses using α-eS26 antibodies. Arc1 served as loading control. (C) eS26 mutants linked to DBA accumulate 20S pre-rRNA in the cytoplasm. $P_{GAL1}$-RPS26Arps26bΔ cells transformed with plasmids encoding for eS26 WT and mutant proteins were grown at 37°C to mid-log phase in glucose containing medium. Localization of 20S pre-rRNA was analyzed by FISH using a Cy3-labeled oligonucleotide complementary to the 5′ portion of ITS1 (red). Nuclear and mitochondrial DNA was stained with DAPI (blue). Scale bar = 5 µm.
Figure 26. The eS26C77W mutant associated with Klippel-Feil syndrome in Diamond-Blackfan anemia patients is impaired in binding importins.

(A) Tsr2 interacts with eS26 mutants linked to DBA. Recombinant GST-Tsr2 was immobilized on Glutathione Sepharose and then incubated with E. coli lysates containing eS26aFLAG, eS26D33NFLAG or eS26C77WFLAG lysates for 1 h at 4°C. Bound proteins were eluted by SDS sample buffer, separated by SDS-PAGE and detected by Coomassie Blue staining. L = input.

(B) eS26C77W is impaired in binding to Kap123, Kap104 and Pse1. Recombinant GST-Kap123, GST-Kap104, GST-Pse1 and GST alone were immobilized on Glutathione Sepharose and then incubated with E.coli lysate containing eS26FLAG, eS26D33NFLAG or eS26C77WFLAG lysates for 1 h at 4°C. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining and Western analyses using α-eS26 antibody. L = input.

(C) The GFP-eS26D33N fusion protein is efficiently targeted to the nucleus. WT cells expressing GFP-eS26 and GFP-eS26D33N were grown in synthetic media at 30°C to mid-log phase and the localization of GFP-eS26 was analyzed by fluorescence microscopy. Scale bar = 5 µm.
weakly with these importins (Figure 26B). We were unable to localize GFP-eS26C77W; whole cells extracts revealed that GFP-eS26C77W protein levels were strongly reduced (Figure S5D).

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MECHANISTIC AND STRUCTURAL INSIGHTS INTO THE ROLE OF THE ESCORTIN TSR2

1. INTRODUCTION

Ribosome biogenesis involves the assembly of more than 70 ribosomal proteins with four different rRNA species and imposes tremendous challenges on eukaryotic cells. The correct folding, stoichiometry and assembly of rRNAs and r-proteins must be constantly assessed to allow further maturation events to occur and to render ribosomes translational-competent (Strunk and Karbstein, 2009). The escortin Tsr2 contributes significantly to the production of translation-competent 40S subunits by promoting a safe transfer of the r-protein eS26 from its nuclear import receptor to its ribosome assembly site in the nucleolus. In the absence of protective Tsr2, the levels of eS26 are drastically reduced due to aggregation and/or degradation of eS26 (Figure 24).

The structure of the eukaryotic-specific ribosomal protein eS26 from *T. thermophila* bound to the mature 40S subunit was solved by X-ray crystallography in 2011 (Figure S4A; Rabl et al., 2011). In eukaryotes, eS26 is bound in a region corresponding to the bacterial anti-Shine-Dalgarno sequence. Through mainly electrostatic interactions, eS26 appears to tightly lock the flexible 3’ end of the 18S rRNA in place, thereby stabilizing helix 45. In addition to 18S rRNA, eS26 interacts with r-proteins uS11 and eS1 on the 40S subunit.

eS26 does not have a compact tertiary structure, and it is predicted to be intrinsically unstable and prone to proteolytic degradation and aggregation in its unbound state. The N-terminus of eS26 is deeply buried within the 40S subunit, whereas the C-terminus is more accessible on the surface (Figure S4A). Both N- and C-termini are mainly unstructured, and eS26 contains a zinc-finger motif, an antiparallel β-sheet and two short α-helices, which are buried within the rRNA framework of the 40S subunit (Figure S6). The surface exposed site of eS26 is composed of an α-helix and an antiparallel β-sheet containing the highly conserved eukaryotic motif YxxPKxYxK. It has been
proposed that this motif is involved in translation initiation by interacting with both the 5’ UTR of mRNAs and the translation initiation factor eIF3 (Sharifulin et al., 2012).

The structure of the eS26 escortin Tsr2 as well as the molecular basis on how Tsr2 protects, stabilizes and escorts eS26 to the maturing pre-ribosome is not yet elucidated. Shedding light on the structure of Tsr2 and the Tsr2:eS26 complex would provide insight into the molecular basis of how an escortin functions in stabilizing and transferring its cargo after nuclear import from its import receptor to its assembly site.

Here we present the structure of the N-terminal domain of the escortin Tsr2 comprising residues 1-152. Tsr2^{1-152} is composed of six tightly packed α-helices and a short two-stranded anti-parallel β-sheet. This domain is sufficient for the interaction between Tsr2 and eS26. Although the C-terminus is not essential for the interaction with eS26, it contributes to eS26 stability and plays an important role in keeping eS26 in an RNA-free state.

2. RESULTS

2.1. eS26 co-purifies RNA

In order to elucidate the molecular basis of the Tsr2 eS26 interaction, we first purified recombinant GST-eS26 from E. coli. To our surprise, we found that purified GST-eS26 shows higher absorption at 260 nm than at 280 nm in a UV absorption spectrum (Figure 27A), indicating that it co-purifies with nucleic acid. We therefore performed the last purification steps of GST-eS26 in presence of RNaseA. However, immediately after RNaseA addition, visible aggregation and precipitation of GST-eS26 was observed (Figure 27B). This indicates that GST-eS26 binds RNA, that the bound RNA to GST-eS26 is accessible to RNaseA and that the RNA stabilizes recombinant GST-eS26. No RNA co-purified with the Tsr2:eS26 complex (data not shown), indicating that Tsr2 prevents eS26 from interacting with RNA. Moreover, we pre-incubated GST-eS26 on Glutathione Sepharose, added increasing amounts of Tsr2 and analyzed the supernatant and the bound fractions for absorption
Figure 27. eS26 co-purifies with RNA.
(A) Recombinant GST-eS26 co-purifies with RNA. UV absorption spectrum of purified recombinant GST-eS26. (B) GST-eS26 precipitates upon RNaseA treatment. Purified GST-eS26 (~100 µM) was supplied with a final concentration of 0.01 µg/mL RNaseA. Immediate precipitation of GST-eS26 was observed. (C) Tsr2 displaces RNA bound to GST-eS26. Increasing amounts of Tsr2 (0.3-6 µM) were added to GST-eS26 immobilized on Glutathion Sepharose. The absorbance at 260 nm of the flowthrough (RNA eluted from GST-eS26) and of the bound fraction (after GSH elution) was determined. (D) Upon binding, Tsr2 can displace the RNA from GST-eS26. Recombinant GST-eS26 and GST were immobilized on Glutathion Sepharose and incubated with increasing amounts of purified Tsr2 (0.7 µM-14 µM) for 1 h at 4°C. RNA content bound to the beads and eluted from the beads by Tsr2 was analyzed on a denaturing ethidiumbromide stained agarose gel. T: total RNA sample from WT yeast cells. M: RNA ladder.
at 260 nm. We found, that upon addition of increasing amounts of Tsr2, RNA was successively eluted from GST-eS26, though the absorbance at 260 nm increased in the flow-through (Figure 27C, grey columns) and decreased on the bound fraction (Figure 27C, blue columns). We analyzed bound fractions and flow-through on a denaturing agarose gel and found that the most abundant RNA species bound to eS26 had an approximate size of 1,500 bp (Figure 27D). Further experiments are needed to identify the RNA bound to GST-eS26 and to determine whether this interaction is specific. The size of the bound RNA is in accordance with the size of 16S rRNA from *E. coli*. However, since eS26 can bind through its YxxPKxYxK motif to 5' UTRs of mRNAs, the co-purifying RNA could also be *E.coli* mRNA.

### 2.2. The N-terminus of Tsr2 is sufficient to interact with eS26

Analysis of the primary sequence of Tsr2 revealed a highly acidic, glutamate and aspartate-rich C-terminal tail, encompassing amino acids 153-205 (Figure 28A). Secondary structure predictions indicate that this acidic tail is unstructured, whereas the N-terminal part of Tsr2 is predicted to be mainly α-helical. We hypothesized that the highly acidic C-terminus of Tsr2 might mimic the 3' end of the 18S rRNA, which binds eS26 once it is incorporated into the 40S subunit. Thus, we would predict that the C-terminal tail maintains non-ribosome bound eS26 in a state devoid of RNA and is the major domain of Tsr2 interacting with the highly basic eS26. To our surprise, yeast two-hybrid analyses revealed no interaction between Tsr2^{153-205} and eS26 (Figure 28B). In contrast, in this assay, the N-terminal domain of Tsr2, Tsr2^{1-152}, strongly interacted with eS26, as determined by growth on stringent adenine deficient media (Figure 28B). To identify the minimal binding motifs in Tsr2, we constructed additional Tsr2 N-terminal domain truncations and analyzed their interactions with eS26 by yeast two-hybrid. This approach identified Tsr2^{1-152} as the minimal region necessary to interact with eS26 (Figure 28C). Furthermore, all tested truncations of eS26 were unable to interact with Tsr2 (Figure 28C), suggesting that both N- and C-terminal regions are important for the interaction and/or that they contribute to the stability of eS26. *In vitro* binding studies using recombinant proteins confirmed that eS26 and Tsr2^{1-152}
Figure 28. Tsr2\(^{1152}\) interacts with eS26.

(A) Tsr2 contains a highly acidic C-terminal tail. Schematic of Tsr2 showing the acidic tail in red and the N-terminus in blue. (B) Tsr2\(^{1152}\) interacts with eS26 in a yeast two-hybrid assay. Plasmids encoding the indicated GAL4 DNA-binding domain (BD) and GAL4 activation domain (AD) fusion proteins were transformed into the yeast reporter strain NMY32. Transformants were spotted in 10-fold serial dilutions onto SDC-Leu-Trp (-Leu-Trp) or SDC-Ade (-Ade) and incubated at 30°C for 4 days. Growth on SDC-Ade indicated a strong two-hybrid interaction. The SV40 Large T antigen served as negative control for these analyses. (C) Tsr2\(^{1152}\) is the minimal motif in Tsr2 interacting with eS26. Schematics summarizing the results from yeast two-hybrid interaction studies performed with truncations of Tsr2 and eS26. (D) Tsr2\(^{1152}\) directly binds eS26 in vitro. GST-eS26 was immobilized on Glutathione Sepharose before incubation with purified His\(_6\)-Tsr2 or His\(_6\)-Tsr2\(^{1152}\). Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining. L= input. Bound Tsr2 and Tsr2\(^{1152}\) are indicated with asterisk.
Figure 29. Tsr2\textsuperscript{1-152} is functional but cannot completely prevent the aggregation of eS26.

(A) Tsr2\textsuperscript{1-152} rescues the slow growth of Tsr2-depleted cells. The P\textsubscript{GAL1}-TSR2 cells transformed with indicated plasmids were spotted in 10-fold dilutions on selective glucose containing plates and grown at indicated temperatures for 3-7 days. (B) Tsr2-TAP co-enriches higher amounts of eS26 than Tsr2\textsuperscript{1-152}-TAP. Tsr2-TAP and Tsr2\textsuperscript{1-152}-TAP were isolated by tandem affinity purification and the Calmodulin eluates were separated in a 4-12% gradient SDS-PAGE and visualized by Silver staining and Western analyses using the indicated antibodies. (C) A molar excess of Tsr2\textsuperscript{1-152} partially prevents aggregation of recombinant eS26 \textit{in vitro}. Aggregation assays were performed 384-well plates. In each well 33 µM GST-eS26 and a given concentration of Tsr2 or Tsr2\textsuperscript{1-152} (0 up to 266 µM) in PBSKMT were pre-incubated for 1 h at 4°C (final volume: 90 µl). 250 nM of PreScission protease was added to initiate proteolysis. After a 1 h incubation, the scattering signal of precipitated eS26 was monitored by measuring the intensity at 450 nm (Y-axis). Concentration of Tsr2 or Tsr2\textsuperscript{1-152} used in the assay (X-axis) are expressed as a molar ratio of eS26:Tsr2/Tsr2\textsuperscript{1-152}. Four replicates for each well were measured. The error bars indicate the standard deviation.
formed a stable complex (Figure 28D) whereas the acidic tail of Tsr2 comprising residues 153-205 did not bind eS26 (Figure 30C). Furthermore, tsr2<sup>1-152</sup> complemented the slow growth phenotype of the P<sub>GAL1</sub>-TSR2 strain on glucose to the same extend as TSR2, indicating that the C-terminal acidic tail is not essential for the function of Tsr2 (Figure 29A). Even though the C-terminus of Tsr2 is not essential for its function, tandem affinity purifications of Tsr2<sup>1-152</sup>-TAP showed a significant decrease in binding to eS26 compared to Tsr2-TAP (Figure 29B). Moreover, Tsr2<sup>1-152</sup> was less able than Tsr2 to suppress the tendency of eS26 to precipitate upon the proteolytic removal of the highly soluble GST-tag (Figure 29C). We conclude that the N-terminal part of Tsr2 is sufficient for its function and its interaction with eS26. Nevertheless, the C-terminus appears to contribute to stabilization and binding to eS26.

### 2.3. The C-terminus of Tsr2 prevents eS26 to interact with RNA

To elucidate the role of the C-terminal tail of Tsr2, we co-expressed and co-purified the Tsr2<sup>1-152</sup>-eS26 complex. Like GST-eS26, the Tsr2<sup>1-152</sup>-eS26 complex co-purified with RNA, since the UV absorption spectrum of the complex displayed an intense maximum at 260 nm (data not shown). In agreement with this data, addition of Tsr2<sup>1-152</sup> to GST-eS26 could only partially replace the bound RNA (Figure 30A) and suppress GST-eS26 aggregation upon RNaseA addition (Figure 30B). These results demonstrate that only full-length Tsr2 containing the C-terminus is capable of displacing RNA from eS26 and that the binding of eS26 to the C-terminal tail of Tsr2 and RNA is mutually exclusive. We wondered whether the C-terminal tail per se is capable of preventing RNaseA induced aggregation of GST-eS26. This domain could only partially prevent RNaseA induced aggregation of GST-eS26 when present in very great excess, as measured by scattering intensity (Figure 30B). However, collective addition of Tsr2<sup>1-152</sup> and Tsr2<sup>153-205</sup> completely prevented aggregation of eS26 in the presence of RNaseA, suggesting that these Tsr2 domains are sufficient in trans to stabilize eS26 devoid of RNA (Figure 30B). In agreement with this result, addition of Tsr2<sup>1-152</sup> to eS26 enabled a weak interaction with GST-Tsr2<sup>153-205</sup> (Figure 30C),
Figure 30. Tsr2<sup>1-152</sup> and Tsr2<sup>153-205</sup> together prevent aggregation and precipitation of recombinant eS26 in vitro.

(A) Tsr2 but not Tsr2<sup>1-152</sup> can displace RNA from GST-eS26. Recombinant GST-eS26 was immobilized on Glutathion Sepharose and incubated with increasing amounts of purified Tsr2 or Tsr2<sup>1-152</sup> (0.3 µM-6 µM) for 1 h at 4°C. After Tsr2 or Tsr2<sup>1-152</sup> addition RNA content bound to the beads was eluted and analyzed by UV absorption at 260 nm. (B) The aggregation assay was performed in a 96-well plate. In each well ~40 µM GST-eS26 and a given concentration of Tsr2, Tsr2<sup>1-152</sup>, Tsr2<sup>153-205</sup> or a combination (0 up to 160 µM) in PBSKMT was pre-incubated for 1 h at 4°C (final volume: 60 µl). 17 µg/ml RNaseA was added to initiate aggregation. After 2 h the scattering signal of the aggregated and precipitated eS26 was monitored by a 96-well plate reader by measuring the intensity at 450 nm (Y-axes). Concentration of Tsr2, Tsr2<sup>1-152</sup> and/or Tsr2<sup>153-205</sup> used in the assay (X-axes) are expressed as a molar ratio of eS26:Tsr2/Tsr2<sup>1-152</sup>/Tsr2<sup>153-205</sup>. Two replicates for each well were measured. The error bars show the standard deviation. (C) Tsr2<sup>153-205</sup> forms a complex with Tsr2<sup>1-152</sup>:eS26. Recombinant GST-Tsr2<sup>153-205</sup> was immobilized on Glutathione Sepharose and then incubated with E. coli lysates containing eS26a<sup>FLAG</sup>, or purified His<sub>6</sub>-Tsr2, His<sub>6</sub>-Tsr2:eS26a<sup>FLAG</sup>, His<sub>6</sub>-Tsr2<sup>1-152</sup> or His<sub>6</sub>-Tsr2<sup>1-152</sup>:eS26a<sup>FLAG</sup> for 1 h both in presence and in absence of 10 µg/mL RNaseA at 4°C. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and detected by Coomassie Blue staining and Western analyses using the indicated antibodies. L= input.

suggesting that the N-terminus of Tsr2 is necessary for an interaction between eS26 and the C-terminus of Tsr2. This indicates that the N-terminus of Tsr2 is either crucial for the C-terminal tail to adopt a conformation capable of binding eS26 or that eS26 is only competent to bind to the acidic C-terminus of Tsr2 in presence of the N-terminus. After RNaseA treatment the interaction between GST-Tsr2<sup>153-205</sup>, eS26 and Tsr2<sup>1-152</sup> was further enhanced (Figure 30C). These results demonstrate that, in contrast to full-length Tsr2, the separate N- and C-terminal parts of Tsr2 require RNA degradation by RNaseA to completely release the eS26 bound RNA and to tightly bind to eS26. Although the acidic C-terminus of Tsr2 is not essential under the tested conditions, we conclude that it strongly contributes to eS26 binding and to keeping eS26 in an RNA-free state.

2.4. The 3’ end of the 18S rRNA is not sufficient to disassemble the Tsr2:eS26 complex

As described in the first part of this thesis, eS26 clamps the 3’ end of the 18S rRNA on the mature ribosome. We wondered whether the 21 terminal nucleotides of the 3’ end of the 18S rRNA are sufficient to trigger eS26 release from Tsr2. However, we found that the addition of RNA to the Tsr2:eS26 complex could not induce complex disassembly (data not shown).
Since the two separated Tsr2 domains might have a lower affinity to eS26 than full-length Tsr2, we wondered whether the 3’ end of 18S rRNA could disassemble eS26 from the trimeric Tsr2\(^{1-152}\).Tsr2\(^{153-205}\).eS26 complex. However, we did not detect a significant release of eS26 from Tsr2\(^{1-152}\).GST-Tsr2\(^{153-205}\) (Figure 31). This data indicates that the 3’ end of the 18S rRNA alone is not sufficient to trigger an efficient release of eS26 from Tsr2.

**Figure 31. The 3’ end of 18S rRNA cannot displace eS26 from Tsr2\(^{1-152}\).Tsr2\(^{153-205}\).** Recombinant GST-Tsr2\(^{153-205}\) was immobilized on Glutathione Sepharose and then incubated with purified His\(_6\)-Tsr2\(^{1-152}\).eS26a\(^{\text{FLAG}}\) complex for 1 h in presence and absence of 10 µg/mL RNaseA at 4°C. Bound proteins were washed PBSKMT and resuspended in PBSKMT containing 10 mM Ribonucleoside Vanadyl Complex. 20 µM 3’ end of the 18S rRNA was added and incubated for 1 h at 4°C. Elution, separation and detection were performed as in (C). L= input.

### 2.5. NMR structure determination of TSR2\(^{1-152}\)

In order to understand the molecular basis of the Tsr2-mediated stabilization of eS26, we aimed to solve the solution structure of Tsr2 and characterize the structural interaction between Tsr2 and eS26. Since the C-terminus of Tsr2 is highly enriched in acidic amino acids and mainly unstructured, we first decided to investigate the structure of the functional domain of Tsr2, Tsr2\(^{1-152}\).

All NMR experiments for both the resonance assignments and the structure determination were recorded using a NMR sample containing 1 mM of Tsr2\(^{1-152}\) in NMR buffer (20 mM sodium phosphate pH 7.0, 50 mM NaCl, 1 mM DTT, 10 µM EDTA, 95% H\(_2\)O/5% \(^2\)H\(_2\)O). The \(^1\)H-\(^{15}\)N HSQC spectrum of Tsr2\(^{1-152}\) shows well-dispersed resonances, indicating that the protein is folded (Figure 32). Based on the 3D heteronuclear NMR experiments and the
3D NOESY spectra we obtained complete $^1\text{H}$, $^{13}\text{C}$ and $^{15}\text{N}$ assignments of Tsr2$^{1-152}$ with the following exceptions: $\beta\text{CH}_2$ and $\gamma\text{CH}_2$ of R67, $\zeta\text{CH}$ of F101 and $\delta^2\text{CH}_3$ of L111.

For structure determination we used a combined procedure consisting of ATNOS peak picking and CYANA NOE assignment and CYANA structure calculation. This approach resulted in the assignment of 2418, 435 and 5880 NOE cross peaks in the 3D $^{15}\text{N}$-resolved $[^1\text{H},^1\text{H}]-\text{NOESY}$ spectrum, the 3D aromatic $^{13}\text{C}$-resolved $[^1\text{H},^1\text{H}]-\text{NOESY}$ spectrum and the 3D aliphatic $^{13}\text{C}$-resolved $[^1\text{H},^1\text{H}]-\text{NOESY}$ spectrum, respectively, yielding 3818 meaningful NOE upper distance limits as input for the CYANA structure determination, which was further supplemented with 521 constraints for backbone and side-chain torsion angles. After energy-minimization of the 20 CYANA conformers in implicit water using the program AMBER12 (Case et al., 2012) we obtained a well-defined structure for Tsr2$^{1-152}$ with an RMSD value of 0.22 Å calculated for the backbone atoms and 0.47 Å for all heavy atoms of the structured region of the protein (residues 10–19, 23–27, 31–54 and 63–136; see Table 3).

Figure 32. $^1\text{H}-^{15}\text{N} \text{HSQC of Tsr2}^{1-152}$. The $^1\text{H}-^{15}\text{N}$ HSQC spectrum of Tsr2$^{1-152}$ was recorded at 293.15 K on a Bruker Avance 500 MHz spectrometer. Tsr2$^{1-152}$ displays well-dispersed signals, indicating that the protein is folded.
Table 3. Input for the structure calculation and characterization of the 20 energy-minimized NMR structures of TSR2\textsuperscript{1-152}

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<th>Parameter</th>
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<td>Ramachandran plot statistics (%)\textsuperscript{c}</td>
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<td>Disallowed regions</td>
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</table>

\textsuperscript{a} Except for the top six entries, the data characterize 20 CYANA conformers after energy-minimization with AMBER that are used to represent the NMR structure; the mean values and standard deviations are given.

\textsuperscript{b} Backbone heavy atoms include N, C\textsuperscript{\textalpha} and C'. The RMSD values are calculated for the structured regions of Tsr2\textsuperscript{1-152}, which include residues 10–19, 23–27, 31–54 and 63–136.

\textsuperscript{c} As determined by PROCHECK.
Figure 33. NMR structure of Tsr$_2^{1-152}$.
Shown is a superposition of the protein backbone of 20 energy-minimized CYANA conformers in ribbon representation. For improved visualization the molecules are also displayed in a 90° rotation around the vertical axis.

The determined NMR solution structure of Tsr$_2^{1-152}$ consists of six tightly packed $\alpha$-helices comprised of residues 32–46 ($\alpha_1$), 48–56 ($\alpha_2$), 63–78 ($\alpha_3$), 86–99 ($\alpha_4$), 110–125 ($\alpha_5$) and 130–143 ($\alpha_6$) and a short two-stranded anti-parallel $\beta$-sheet comprised of residues 15–17 ($\beta_1$) and 83–85 ($\beta_2$) (Figure 33). The loops connecting the secondary structure elements are well defined.

Figure 34. Heteronuclear NOE experiment.
Heteronuclear $^{15}$N-$^1$H nuclear Overhauser effect (NOE) experiment of TSR2$_1^{1-152}$ recorded at 293.15 K and 700 MHz with a relaxation delay of 5 seconds. The resulting NOE values are derived from the ratio of peak amplitudes of individual amide resonances from experiments recorded with and without proton saturation. The data show that the amide bond vectors of the termini display increased motion and that the core of the protein forms a compact and rigid fold.
The heteronuclear NOE (HetNOE) experiment indicated that the protein forms a compact structure in which only the terminal residues display increased structural flexibility (Figure 33 and Figure 34). The loops between \( \beta_1 \) and \( \alpha_1 \) as well as the loop between helices \( \alpha_4 \) and \( \alpha_5 \) display slightly increased structural mobility. The decreased HetNOE value of Trp69 compared to its neighboring residues likely arises from a signal overlap with the amide resonance of the highly mobile His153.
Figure S1. Tsr2 and eS26 depletion does not impair pre-40S nuclear export.

(A) Sequence alignment of Tsr2 from the indicated organisms done by ClustalO (Sievers and Higgins, 2014; Sievers et al., 2011). Conservation at each position is depicted as a gradient from light blue (50% identity) to dark blue (100% identity). (B) Tsr2- and eS26-depletion does not impair pre-40S subunit nuclear export. The indicated strains expressing uS5-GFP were grown in repressive glucose containing liquid media to mid-log phase at 30˚C. Localization of uS5-GFP was monitored by fluorescence microscopy. Scale bar = 5 µm. (C) Human Tsr2 rescues the slow growth of Tsr2-depleted cells. The $P_{GAL1}$-TSR2 cells transformed with indicated plasmids were spotted in 10-fold dilutions on selective glucose containing plates and grown at indicated temperatures for 3-7 days.
Figure S2. Tsr2 and eS26 protein levels in the indicated TAP strains and levels of 20S pre-rRNA and 18S rRNA in the indicated TAP purified particles.

(A) Noc4-, Enp1- and Rio2-TAP purify pre-40S subunits containing immature 20S pre-rRNA whereas Asc1-TAP purifies a 40S subunit containing mature 18S rRNA. 1 µg of RNA isolated from the indicated pre-40S TAP-eluates was separated on a 2% Agarose gel and probed against indicated rRNAs by Northern blotting. 1 µg of total RNA extracted from WT cells was used as a control.

(B) eS26 does not co-enrich with the earliest 60S pre-ribosome. Noc4-TAP, the earliest pre-ribosomal particle and Ssf1-TAP, the earliest pre-ribosome in the 60S maturation pathway were isolated. The Calmodulin eluates were visualized by Silver staining and by Western analyses using the indicated antibodies. The CBP signal served as loading controls for the TAPs.

(C) Tsr2 and eS26 protein levels in indicated TAP strains (also used in Figure 20A) are equal to levels in WT cells. Whole cell extracts (WCE) were prepared from the indicated strains and analyzed by Western analyses using antibodies against Tsr2 and eS26. The protein Arc1 served as loading control.
Figure S3. eS26, but not Tsr2:eS26 or Tsr2, interacts with importins.
Recombinant GST tagged importins, immobilized on Glutathione Sepharose, were incubated with purified 3.4 µM Tsr2, 4 µM Tsr2:eS26 or E. coli lysate containing ~4 µM eS26^FLAG in PBSKMT and competing E. coli lysates for 1 h at 4°C. After washing, bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE, and visualized by either Coomassie Blue staining or Western analyses using indicated antibodies. L= input. GST-tagged importins are indicated with asterisk, His^6^-Srp1 is indicated with a rectangle.
Figure S4. GFP-eS26 binds to importins and Tsr2 but is not incorporated into pre-ribosomes.

(A) Location of N- and C-terminus of eS26 within the mature 40S subunit (Rabl et al., 2011). eS26 N-terminus (green) is embedded deeply within the 40S subunit whereas the C-terminus (red) projects away from the body of the 40S subunit. Red letters indicate the 20 C-terminal residues that are not visualized in the structure (B) GFP-eS26 is not found in heavier fractions on sucrose gradients. WT lysates and lysates containing GFP-eS26 were subjected to sucrose gradient sedimentation as described in Figure 17B. The peaks for 40S and 60S subunits, 80S ribosomes and polysomes are indicated. The proteins in the gradient were detected by Western analyses using the indicated antibodies. (C) GFP-eS26 binds to Kap123, Kap104 and Pse1. Recombinant GST-Kap123, -Kap104, -Pse1 and GST alone were immobilized on Glutathione Sepharose and then incubated with E.coli lysate containing GFP-eS26 for 1 h at 4°C. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining and Western analyses using α-GFP antibody. L= input.
Figure S5. GFP-eS26 is not functional but is imported into the nucleus.

(A) GFP-eS26 is unable to rescue the lethality of the eS26 deficient strain. The P_{\text{GAL1}}-\text{RPS26A}rps26bΔ strain transformed with different plasmids encoding eS26 or GFP-eS26 were spotted in 10-fold dilutions on selective glucose containing plates and grown at indicated temperatures for 3-7 days. (B) GFP-eS26 and GFP-eS26D33N levels are strongly reduced in Tsr2-depleted cells. Whole cell extracts (WCE) prepared from WT and Tsr2-depleted cells were assessed by Western analyses using antibodies against the indicated proteins. Arc1 protein levels served as loading control. (C) Upper panel: The Zn^{2+}-binding domain of eS26 is required for efficient nuclear uptake. WT cells expressing GFP-eS26 truncations were grown in synthetic media at 30°C to mid-log phase and the localization of GFP-eS26 truncations was analyzed by fluorescence microscopy. Scale bar = 5 μm. Lower panel: Schematic for the eS26 truncations used for fluorescence microscopy. (D) GFP-eS26C77W protein levels are strongly reduced in (WCE) extracts. Whole cell extracts were prepared from P_{\text{GAL1}}-\text{RPS26A}rps26bΔ cells transformed with plasmids encoding for GFP-eS26 WT and mutant proteins. eS26 protein levels were assessed by Western analyses using α-\text{GFP} antibodies. Arc1 served as loading control.
Figure S6. eS26 is conserved and binds in the mature ribosome to the 3’ end of 18S rRNA.

Upper panel: Sequence alignment of eS26 from the indicated organisms done by ClustalO (Sievers and Higgins, 2014; Sievers et al., 2011). Conservation at each position is depicted as a gradient from light blue (50% identity) to dark blue (100% identity). Mutated residues linked to DBA are depicted with orange (Asp33) and green (Cys77) dots.

Lower panel: Location of eS26 within the mature 40S subunit (Rabl et al., 2011). eS26 clamps the 3’ end of the mature 18S rRNA at the site where the endonuclease Nob1 cleaves the immature 20S pre-rRNA. Inset depicts the 3’ end portion of 18S rRNA (red) in contact with eS26 (blue). The position of amino acids D33 (orange) and C77 (green) that are mutated in DBA or KFS and the coordinated Zn$^{2+}$ ion (black) are depicted.
Figure S7. The 3’ end of 18S rRNA does not facilitate dissociation of the Kap123:eS26 complex by RanGTP.

(A) RanGTP and the 3’ end of 18S rRNA cannot dissociate the Kap123:eS26 complex. GST-Kap123:eS26aFLAG complexes, immobilized on Glutathione Sepharose, were incubated with buffer alone or with 1.5 µM RanGTP, 1.5 µM Tsr2, 3 nM of the 3’ end of 18S rRNA or the combination of RanGTP and the 3’ end of 18S rRNA for 1 h at 4°C. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie Blue staining and Western analyses using α-eS26 antibodies. L = input. GST-tagged importins are indicated with asterisks.

(B) RanGTP, but not Tsr2 dissociated the Pse1:Slx9 complex in vitro. Pse1:Slx9 complexes were immobilized on Glutathione Sepharose and incubated with buffer alone or with 1.5 µM RanGTP, 1.5 µM Tsr2 or 3 nM 3’ end of 18S rRNA for 1 h at 4°C and analyzed as in Figure 21A. GST-tagged importins are indicated with asterisks.
Figure S8. RanGTP and Tsr2 do not release eS31, eS8 and uS14 from Kap123.

GST-Kap123 and GST alone were immobilized on Glutathione Sepharose and incubated with E. coli lysate containing ~4 µM eS14\textsuperscript{FLAG}, eS31\textsuperscript{FLAG} or eS8\textsuperscript{FLAG} in PBSKMT combined with competing E. coli lysates for 1 h at 4°C. GST-Kap123:eS14\textsuperscript{FLAG}, GST-Kap123:eS31\textsuperscript{FLAG}, GST-Kap123:eS8\textsuperscript{FLAG} complexes were incubated with either buffer alone or with 1.5 µM RanGTP or 1.5 µM Tsr2 for 1 h at 4°C. Bound proteins were eluted in SDS sample buffer and separated by SDS-PAGE. Proteins were visualized by Coomassie Blue staining or Western analyses using α-FLAG-antibodies. L= input. GST-Kap123 is indicated with asterisks.
Figure S9. Tsr2 efficiently dissociates importin:eS26\textsuperscript{FLAG} complexes.
GST-Kap104: eS26\textsuperscript{FLAG} and GST-Pse1:eS26\textsuperscript{FLAG} complexes immobilized on Glutathione Sepharose were incubated with either buffer alone or with 1.5 µM or 375 nM RanGTP or 1.5 µM or 375 nM Tsr2. Samples were withdrawn at the indicated time points (1, 2, 4, 8 min). Washing, elution, and visualization were performed as in Figure 21A. GST-tagged importins are indicated with asterisks.

![GST-Tsr2/S26A GST](image)

Figure S10. The Tsr2:eS26 complex is not disassembled by importin Kap123.
GST-Tsr2:eS26 was immobilized on Glutathione Sepharose and incubated with \textit{E. coli} lysate containing His\textsubscript{6}-Kap123 combined with competing \textit{E. coli} lysates for 1 h at 4°C. Bound proteins were eluted in SDS sample buffer and separated by SDS-PAGE. Proteins were visualized by Coomassie Blue staining. L= input.

![Figure S10](image)

Figure S11. eS26 serine mutants are impaired in interacting with Tsr2 in a yeast two-hybrid assay.
Plasmids encoding the indicated GAL4 DNA-binding domain (BD) and GAL4 activation domain (AD) fusion proteins were transformed into the yeast reporter strain NMY32. Transformants were spotted in 10-fold serial dilutions onto SDC-Leu-Trp (-Leu-Trp), SDC-His (-His) or SDC-Ade (-Ade) and incubated at 30°C for 4 days. Growth on SDC-His indicates a weak, growth on SDC-Ade a strong yeast two-hybrid interaction. The SV40 Large T antigen served as negative control.
Figure S12. The incorporation of eS26 in early pre-ribosomal particles is salt dependent.
Pre-ribosomal particles of the 40S maturation pathway were purified in TAP lysis buffer containing indicated concentrations of NaCl using Enp1- and Asc1-TAP as baits. Calmodulin-eluates were analyzed by Western analyses using the indicated antibodies. The r-protein uS7 and CBP served as loading controls for the TAPs.

Figure S13. Importins are not the sole cytoplasmic chaperones for eS26. (A) eS26 protein levels are not reduced in importin mutant strains. eS26 protein levels in whole cell extracts derived from the indicated strains were determined by Western analyses using α-eS26 antibodies. Protein levels of Arc1 served as loading control. (B) Recombinant Kap123 does not prevent the aggregation of eS26 in vitro. E. coli lysate containing recombinant eS26 was incubated with either buffer alone or with excess of Tsr2 or GST-Kap123 at 37°C in native yeast lysate. Samples were withdrawn at the indicated time points. Aggregated proteins were removed by centrifugation and soluble proteins were TCA precipitated and eS26 levels visualized by Western analyses.
Figure S14. The DBA linked human Tsr2E64G mutant is unable to fully rescue the growth defect of Tsr2-depleted cells. The $P_{\text{GAL}}$-TSR2 strain transformed with plasmids encoding yeast and human TSR2 and TSR2 mutants were spotted in 10-fold dilutions on selective glucose containing plates and grown at indicated temperatures for 3-7 days.
DISCUSSION AND OUTLOOK

From Schütz et al., 2014.

A growing yeast cell manufactures ~200,000 ribosomes during one generation time (Warner, 1999). This process requires the import of ~14 million r-proteins into the nucleus through ~200 NPCs. Such a process entails rapid transport of importin:r-protein complexes into the nucleus, and necessitates an efficient mechanism to dissociate these complexes to terminate the import process. This permits rapid recycling of importins back to the cytoplasm for subsequent rounds of import. Although it is recognized that r-proteins employ multiple import pathways to reach the nuclear compartment, it remains unclear how these intrinsically unstable and aggregation-prone proteins are targeted to the assembling pre-ribosome. It is assumed that, like a typical import cargo, RanGTP releases the r-protein from the importin and the r-protein somehow finds its way to its cognate rRNA site. Here, we reveal that Tsr2 extracts eS26 from importins and ensures its safe transfer to the 90S pre-ribosome. These data implicate an atypical RanGTP-independent mechanism that terminates the import process, and uncovers an unanticipated link between the nuclear import machinery and the ribosome assembly pathway.

1. THE JOURNEY OF THE R-PROTEIN eS26 TO THE MATURING RIBOSOME

1.1. eS26 is recruited to the 90S pre-ribosome

Adapted from Schütz et al., 2014.

Using Western analyses and targeted SRM assays, we found that untagged eS26 is recruited to Noc4-TAP and co-enriches with nuclear pre-40S subunits that contain 20S pre-rRNA (Figure 20A, Figure 20B and Figure S2A). Moreover, eS26-GFP accumulated in the nucleus of yrb2Δ cells that are specifically impaired in 40S pre-ribosome export (Figure 20C). These data suggest that eS26 can be transported to the 90S pre-ribosome. Our findings contrast a previous report wherein a FLAG-tagged eS26 immunoprecipitated
mainly 18S rRNA, suggesting that eS26 is incorporated into pre-40S subunits in the cytoplasm after 20S pre-rRNA processing occurred (Ferreira-Cerca et al., 2007). Although the precise timing of eS26 recruitment remains unclear, based on our data, we propose that it is a late event during 90S assembly.

Our data indicate that eS26 does not affect 90S assembly, nucleoplasmic maturation or proofreading, but instead is specifically required for final 40S subunit maturation (Figure 19B). How can the early nucleolar incorporation of eS26 affect final cytoplasmic 40S maturations? Pre-40S subunits that lack eS26 escape nuclear proofreading and are efficiently transported into the cytoplasm. In the cytoplasm, these incompletely assembled pre-40S subunits, devoid of eS26, are still capable of recruiting the endonuclease Nob1 (Figure 24A) and of forming 80S-like particles (Figure 17B and Figure 19C), which are crucial for final cytoplasmic 40S maturation and 20S pre-rRNA processing. The presence of 80S-like particles in eS26-depleted cells suggests that all factors necessary to mimic a translation-like state are present on this pre-40S subunit. However, despite the formation of 80S-like particles, final 20S pre-rRNA cleavage does not occur. Thus, 20S pre-rRNA within an 80S-like particle becomes an optimal substrate for Nob1 only when the pre-40S subunit has satisfied a checklist that assesses its potential to translate, including the incorporation of eS26. We propose that the cytoplasmic 20S pre-rRNA cleavage functions as one of the checkpoints that prevent incompletely assembled, pre-40S subunits from entering translation.

Why does the absence of eS26 on 80S-like particles impair 20S pre-rRNA processing? In the mature 40S subunit, eS26 keeps the 3’ end of the 18S rRNA tightly locked, precisely at the site where the endonuclease Nob1 cleaves the 20S pre-rRNA. Thus, eS26 might pose restrictions to the conformational flexibility of the 20S pre-rRNA, which could be important for its processing in 80S-like particles. Since Nob1 recruitment is not significantly reduced when eS26 is depleted from pre-40S subunits, a loose 3’ end of the 20S pre-rRNA might indeed hinder pre-rRNA processing to occur within 80S-like particles.

Since eS26 was previously not identified in mass spectrometric studies of a pre-40S subunit, it was suggested that eS26 replaces the assembly factor
Pno1 in the cytoplasm after 20S rRNA processing occurred (Strunk et al., 2011; Strunk et al., 2012). However, we found that eS26 incorporation is crucial for 20S pre-rRNA processing and that Pno1-TAP efficiently co-enriched eS26 (Figure 20A). Further, Tsr2-depletion even impaired recruitment of Pno1 to pre-40S subunits. This indicates that instead of being a placeholder for eS26, the recruitment of Pno1 to the pre-40S seems to be dependent on eS26 incorporation (Figure 24A). Pno1 is assumed to be a regulator of Nob1 activity. The absence of Pno1 on 80S-like particles devoid of eS26 might impair Nob1 activity and impede 20S pre-rRNA processing.

The fact that 80S-like ribosomes can be formed in absence of eS26 demonstrates that pre-40S subunits devoid of eS26 have the capacity to interact with mature 60S subunits. Thus, after 80S-like particle formation, additional mechanisms might exist that hinder pre-mature translation initiation and eradicate incomplete pre-ribosomes from the translating pool. During translation initiation, eS26 is involved in binding the translation initiation factor eIF3 and the 5' UTR of mRNAs (Sharifulin et al., 2012). 40S subunits devoid of eS26 might be unable to recruit eIF3 and to initiate translation. These subunits would then remain in the cytoplasm and might be targeted by quality control mechanisms, leading to exosomal and proteasomal degradation of the rRNA and r-proteins, respectively.

In addition, to prevent 20S pre-rRNA maturation of incompletely assembled pre-40S subunits, eS26 might be implicated in a second cytoplasmic checkpoint, which assesses the capacity of 40S subunits to bind translation initiation factors.

1.2. Nuclear import of eS26
Adapted from Schütz et al., 2014.

Most r-proteins are very small and might therefore be capable of free diffusion into the nucleoplasm. However, most free ribosomal proteins in the cytoplasm are degraded within 2-3 minutes (Warner, 1989; Warner et al., 1985) and are therefore likely to depend on efficient and active nuclear import. For a long time, eS26 was thought to be incorporated into late pre-40S
subunits in the cytoplasm (Ferreira-Cerca et al., 2007; Strunk et al., 2011; Strunk et al., 2012). However, our results demonstrate that eS26 is incorporated into 90S pre-ribosomes in the nucleolus, raising the question of how eS26 is transported into the nucleus. Unlike the Kap104 adaptor Syo1 that co-imports uL18 (yeast Rpl5) and uL5 (yeast Rpl11) (Kressler et al., 2012a), Tsr2 does not mediate interactions between eS26 and importins. Instead, our data identified Kap123 and Kap104 as the major importins that directly bind and transport eS26 into the nucleus (Figure 21). Why are redundant import pathways necessary to import eS26? Kap123 is the major importin for r-proteins (Rout et al., 1997; Schlenstedt et al., 1997), whereas Kap104 is implicated in the transport of many mRNA binding proteins (Aitchison et al., 1996; Lange et al., 2008; Suel and Chook, 2009). Kap104 plays a redundant role with Kap123 in importing eS26, which was not surprisingly, since eS26 is capable of binding the 5’ UTRs of mRNAs (Sharifulin et al., 2012). Moreover, eS26 is an essential protein whereas Kap123 and Kap104 are not, indicating that eS26 import cannot completely depend on only one of the two importins. In in vitro binding assays, eS26 was found to interact strongly with the essential importin Pse1. However, eS26 was not mislocalized to the cytoplasm in a pse1 mutant strain. Hence, Pse1 might play an ancillary role in eS26 import but might be an important backup for eS26 nuclear import under conditions where the other two importins are disabled or titrated by other cargoes. Because rapidly growing yeast cells import up to 3000 r-proteins per second, a redundant import system might be required to provide the capacity and flexibility necessary to coordinate these import processes.

How is eS26 recognized by its transport receptors? Kap104 is known to bind to PY-NLS containing cargoes. However sequence analysis of eS26 revealed no PY-NLS sequence. Moreover, only within a few r-proteins the NLSs interacting with Kap123 could be identified (Moreland et al., 1985; Schaap et al., 1991; Underwood and Fried, 1990). A general NLS motif in Kap123 substrates has not been identified, making identification of the eS26 NLS difficult. However, our data indicate that the NLS in eS26 lies within residues 20-80 enclosing the conserved zinc-finger motif (Figure S5C).
Moreover, the eS26C77W mutant interacted poorly with its import receptors. Cysteine 77 is one of four conserved cysteines within eS26 that coordinates the Zn$^{2+}$ ion (Figure S6, lower panel; Rabl et al., 2011). Our data raise an intriguing possibility that the NLS within eS26 becomes available to interact with importins only when the Zn$^{2+}$ ion is correctly coordinated. In addition to their transport role, importins may select correctly folded eS26.

Structural analyses of the importin:eS26 complexes might shed light on the eS26 NLS and how it binds to importins.

1.3. RanGTP-independent dissociation of importin:eS26 complexes by Tsr2

Adapted from Schütz et al., 2014.

Upon arrival in the nucleoplasm, importin:cargo complexes are usually dissociated by RanGTP. However, recruitment of RanGTP did not efficiently trigger the dissociation of importin:eS26 complexes (Figure 22). One possibility could be that eS26 engages in a novel interaction with the importins, thereby delaying its release. Such a delay may ensure the coordinated handover to the next binding factor, Tsr2. Structural analyses of the importin:eS26 complex should provide clues into why eS26 is inefficiently released from importins by RanGTP.

In contrast to RanGTP, Tsr2 efficiently removed eS26 from its importins (Figure 22 and Figure S9), identifying an atypical RanGTP-independent mechanism to terminate the import cycle. Since Tsr2 does not directly bind to importins, it might not trigger a conformational change in the importin that induces cargo release. Once bound to Tsr2, the addition of increasing amounts of importin did not cause the release of eS26 from Tsr2 and subsequent importin binding (Figure S10). It is likely that Tsr2 binds to importin-bound eS26 via an allosteric mechanism. Structural rearrangements within eS26 might shift the binding equilibrium towards the Tsr2 bound state. Structural information on both the eS26:Tsr2 and eS26:importin complexes might clarify how eS26 is bound to the importin and suggest a mechanism of release by Tsr2.
1.4. How does Tsr2 stabilize eS26?

Adapted from Schütz et al., 2014.

The observation that Tsr2 prevents proteolysis and aggregation of eS26 (Figure 24) indicates an additional “private” chaperone function. Thus our study adds Tsr2:eS26 to the growing list of known chaperones:protein pairs (Sqt1:uL16; Rrb1:uL3; Yar1:uS3) required for ribosome assembly (Eisinger et al., 1997; Iouk et al., 2001; Koch et al., 2012; Schaper et al., 2001).

Our data demonstrate that the N-terminal domain of Tsr2 without the acidic C-terminal tail is sufficient to bind eS26. Although the N-terminal domain is sufficient to prevent aggregation of eS26, the presence of the C-terminus improves its function in stabilizing eS26. The highly acidic C-terminus of Tsr2 might contribute to eS26 stability through electrostatic interactions. Large basic patches in eS26 that might undergo electrostatic interactions are present at its rRNA binding interface and in a small basic patch located close to the α-helix on the solvent exposed part of eS26 (Figure 35A). Whether Tsr2<sup>1-152</sup> contributes to eS26 stability primarily through electrostatic or hydrophobic interactions or a combination of both interactions remains to be elucidated. Since Tsr2<sup>1-152</sup> contains both charged as well as hydrophobic surface patches (Figure 35C), both types of interaction could effectively contribute to eS26 stabilization. Hydrophobic patches in eS26 are present on its ribosome-facing side and seem to be involved in interactions with uS11 (Figure 35B). Hence, hydrophobic interactions between Tsr2<sup>1-152</sup> and eS26 could provide one simple explanation for the observation that eS26:Tsr2 and eS26:40S binding is mutually exclusive.

Whether structural rearrangements occur within the loops or the helices of Tsr2<sup>1-152</sup>, whether the C-terminus adopts a different conformation upon binding or whether eS26 undergoes structural changes needs be clarified. Since the C-terminus of Tsr2 is not essential, it is tempting to speculate that it is not actively involved in releasing eS26 from the importin or in transferring it to the 90S pre-ribosome. It might rather have an alternative function, as the C-terminus is crucial for the displacement of RNA from eS26.
Figure 35. Surface representations of Tsr2\textsuperscript{1-152} and eS26. 
(A) Surface representation of ribosome bound eS26 with electrostatic potential and bound 18S rRNA (grey/silver). (B) Surface representation of the ribosome-facing site of eS26 with electrostatic potentials and uS11 (light blue) (Rabl et al., 2011). (C) Surface representation of Tsr2\textsuperscript{1-152} with electrostatic potentials colored in blue (positive, basic residues), red (negative, acidic) and white (polar and hydrophobic). Tsr2\textsuperscript{1-152} is rotated for 180° around the x-axis for better surface visualization.

Thereby, Tsr2 may prevent eS26 from undergoing non-specific interactions with nucleic acids during its journey towards the 90S pre-ribosome. Whether the C-terminus binds directly to the rRNA binding pocket within eS26 or whether it induces a conformational change within eS26 that triggers the release of non-specific RNA and makes eS26 competent to bind rRNA is still elusive. Moreover, cooperative movements within Tsr2 might be necessary to position the C-terminal tail on eS26 to displace the non-specific RNA. The non-essential nature of the C-terminus suggests that, under normal conditions, eS26 either does not bind to RNA (further discussed in: Imbalanced eS26 levels and ribosomal stress) or that this RNA does not necessarily need to be actively dissociated from eS26. The latter might
indicate that this RNA can adopt the additional stabilizing function of the C-terminus. Moreover, additional mechanisms might exist that act in a redundant way to the C-terminus of Tsr2 and either prevent eS26 from undergoing non-specific interactions with RNA or that displaces non-specifically bound RNA from eS26 prior to its incorporation into 90S pre-ribosomes.

Currently, we are attempting to solve the NMR structure of the Tsr2:eS26 complex, which will provide more insights into how Tsr2 stabilizes eS26. Backbone assignments of Tsr2 in the complex and analysis of the chemical shift perturbations between free and bound Tsr2 will reveal important residues for the interaction with eS26. The structure of eS26 within this complex will elucidate whether Tsr2 brings eS26 in a conformation competent to bind pre-ribosomes and will aid our understanding of how eS26 is incorporated into 90S pre-ribosomes.

1.5. How is eS26 incorporated into 90S pre-ribosomes?

Our data demonstrate that Tsr2 releases eS26 in a RanGTP-independent manner from its transport receptor. However, we still do not understand how eS26 is released from Tsr2 and incorporated into 90S pre-ribosomes. Since the Tsr2:eS26 complex is very stable and the 3’ end of 18S rRNA is not sufficient to release eS26 from Tsr2 in vitro, it is tempting to speculate that post-translational modifications and/or energy consuming enzymes couple the extraction of eS26 from Tsr2 and its subsequent incorporation. eS26 contains a known phosphorylation site (serine 54) which is a putative target to regulate eS26 incorporation. Indeed, we found that mutation of serine 54 and serine 57 reduce the strength of Tsr2 binding, in a yeast-two hybrid approach (Figure S11). However, mutations of the two serine residues to alanine or glutamate residues (phosphomimetic) resulted in the same phenotype, indicating that these serine residues could be important in forming electrostatic interactions with Tsr2. The effects of eS26 phosphorylation and/or dephosphorylation on its interaction with Tsr2 and on incorporation into the 90S pre-ribosome need to be investigated further. The
kinase Hrr25 and the ATPases/kinases Rio1, Rio2, involved in 40S biogenesis, might be implicated in putative phosphorylation events. However, other post-translation modifications might also be involved in regulating the eS26:Tsr2 complex disassembly.

Within the 40S subunit, eS26 closely interacts with uS11. The incorporation of uS11 into the pre-40S subunit is thought to be regulated by the ATPase Fap7. Like Tsr2, Fap7 presumably has a function in protecting the highly basic uS11 from aggregation and the formation of premature or nonspecific interactions with cellular RNAs (Hellmich et al., 2013). Fap7 directly binds uS11 with high affinity and masks its RNA interaction motifs. In its ADP bound state, Fap7 has a high affinity for uS11. It is predicted that upon exchange of ADP to ATP, the C-terminal tail of uS11 is exposed and binds to rRNA. Moreover, Fap7 binding to Nob1 inhibits D-site cleavage (Hellmich et al., 2013). It has been proposed that the final stable incorporation of uS11, involving interactions between its C-terminus and rRNA, occurs at the very last stages of 40S maturation (Jakovljevic et al., 2004). EM structures indicate that uS11 is present on the platform at earlier maturation stages, but not yet at its final position. Fap7 might regulate the stable incorporation of uS11, keeping it soluble before its incorporation, and trigger D-site cleavage. Interestingly, in absence of Fap7, the levels of eS26 and eS1 (yeast Rps1) as well as uS11 are reduced on ribosomes (Strunk et al., 2012). This suggests that either incorporation of uS11 is a prerequisite for eS26 and eS1 incorporation or that Fap7 is also involved in the incorporation of eS26 and eS1. Our data demonstrated that eS26 is already incorporated into the earliest 90S pre-ribosome in the nucleolus (Figure 20). Intriguingly, eS26 incorporation into early pre-40S particles (Enp1-TAP) is salt dependent, whereas in mature particles (Asc1-TAP), in which 20S pre-rRNA processing has occurred, its incorporation is salt-independent (Figure S12). This suggests that the final stable incorporation of uS11 is a prerequisite for stable eS26 incorporation. Might Fap7 also be involved in triggering the release of eS26 from Tsr2? Tsr2 has a motif in its C-terminus that is very similar to the C-terminus of uS11, which is important for Fap7 binding and 20S pre-rRNA
processing. Further experiments will determine whether Tsr2 binds Fap7 and whether this binding triggers eS26 release.

1.6. A model for eS26 incorporation

From Schütz et al., 2014.

Based on our data, we propose a model in which eS26 is transported to the nuclear compartment predominantly by importins Kap123 and Kap104 (Figure 36). Inside the nucleus, eS26 is removed from its importins in a RanGTP-independent mechanism mediated by Tsr2. The released eS26 forms a stable complex with Tsr2. After Tsr2:eS26 complex formation, Tsr2 guarantees a safe transfer of eS26 to the 90S pre-ribosome.

![Figure 36. A model for the transport of eS26 to the 90S pre-ribosome.](image)

Newly synthesized eS26 is transported from the cytoplasm into the nucleus by importins. In the nucleus, Tsr2 alone removes eS26 from importins by a RanGTP-independent mechanism. Subsequently, Tsr2 binds the released eS26, protects it from proteolysis and aggregation, and enables safe transfer to the 90S pre-ribosome. If eS26 is released from the importin by RanGTP it may not immediately encounter Tsr2, resulting in a smaller fraction reaching the 90S pre-ribosome. See Discussion for details of the proposed model. From Schütz et al., 2014.
Although RanGTP is able to inefficiently release eS26 from its importin, failure to immediately bind Tsr2 results in eS26 degradation. Therefore, in absence of Tsr2, only a smaller fraction of eS26 may reach the 90S pre-ribosome, providing a possible explanation as to why Tsr2-deficient cells are severely impaired in growth but are still viable, although the r-protein eS26 is essential. Notably, human Tsr2 can rescue the severe growth defect of the Tsr2-depleted strain (Figure S1C), strongly suggesting an evolutionarily conserved role of Tsr2 in 40S assembly.

1.7. How is eS26 stabilized in the cytoplasm?

Tsr2 localizes primarily to the nucleus, where it protects eS26 from aggregation, degradation and non-specific interactions with RNA. However, since eS26 is largely unstructured and highly basic, cytoplasmic chaperones might also be required co-translationally or immediately after translation of eS26. In kap123Δ and kap104Δ mutant strains, eS26 protein levels are not affected despite the fact that eS26 is mislocalized to the cytoplasm. Since Tsr2 seems also to be imported into the nucleus by Kap123 and mislocalizes to the cytoplasm in a kap123Δ strain, cytoplasmic Tsr2:eS26 co-localization might account for eS26 stabilization. However, in a kap104Δ mutant strain Tsr2 is nuclear and eS26 is cytoplasmic, suggesting that Tsr2 is not the only protein capable of performing a protective function on eS26 (Figure S13A). In addition to their roles as nuclear transport receptors, importins also function as cytoplasmic chaperones for their cargoes (Jäkel et al., 2002). However, using an in vitro degradation assay, we found that recombinant Kap123 was not capable of preventing eS26 aggregation in the presence of native yeast lysates (Figure S13B). Whether Pse1 and/or Kap104 are capable of protecting eS26 from aggregation remains elusive. However, it is unlikely that Tsr2 and importins are the only factors that protect eS26 in vivo during its journey towards the 90S pre-ribosome. After emerging from the translating ribosome, it appears that newly synthesized eS26 is stabilized by yet unknown cytoplasmic factors before being bound by importins. Intriguingly, yeast deficient for the ribosome associated NAC and SSB-RAC chaperone systems that associate with newly synthesized polypeptides accumulate eS26
in ribosomal protein aggregates (Koplin et al., 2010). We speculate that these chaperone systems protect eS26 in the cytoplasm and transfer it to its transport receptors, which mediate nuclear import.

1.8. Imbalanced eS26 levels and ribosomal stress

We have demonstrated that E. coli purified GST-eS26 binds to RNA (Figure 27). However the identity and specificity of this RNA interaction is unknown and needs to be further investigated. Does this RNA:eS26 interaction also occur in yeast and has it a functional relevance? On the ribosome, eS26 is part of the platform responsible for mRNA binding. Whether only ribosome-bound eS26 or also free eS26 is capable to bind mRNA is unclear.

Our data indicate that Tsr2 has the capacity to release bound RNA from eS26 upon complex formation (Figure 27C and Figure 27D). Since Tsr2 is mainly nuclear, it might be involved in releasing putative RNAs from eS26 or preventing eS26 from interacting with RNAs only after nuclear import. Interestingly, human eS26, which is highly homologous to yeast eS26, binds to intron I, to the 5’ UTR and the coding region of its own pre-mRNA and thereby inhibits its splicing (Ivanov et al., 2004). The yeast eS26 pre-mRNA also contains an intron in the 5’ UTR, suggesting that its splicing could also be regulated through eS26 binding. In general, yeast pre-mRNAs encoding r-proteins are the most abundant substrates for splicing (Ares et al., 1999; Spingola et al., 1999). Most introns in r-protein pre-mRNAs are necessary for optimal cell fitness and growth under stress and are regulators of ribosome biogenesis and function (Parenteau et al., 2011). It is known that the yeast protein eL30 (yeast Rpl30) regulates its abundance by an autoregulatory feedback mechanism (Vilardell et al., 2000a). Under normal conditions, eL30 is rapidly incorporated into ribosomes. The RPL30 pre-mRNA is efficiently spliced, exported into the cytoplasm and translated. An imbalance in ribosome biogenesis might lead to increased eL30 levels. Under these conditions, eL30 is imported into the nucleus, but is not incorporated into pre-ribosomes. Instead, it binds to its pre-mRNA an inhibits its splicing (Vilardell et al.,
Unspliced pre-mRNAs bound to eL30 are proposed to be exported into the cytoplasm, where they initiate translation but trigger the nonsense mediated decay mRNA surveillance pathway. Furthermore, eL30 is capable of binding its mature mRNA, thereby inhibiting its translation (Dabeva and Warner, 1993) and adding an additional regulatory mechanism. It is tempting to speculate that a similar autoinhibitory mechanism might exist to regulate eS26 protein levels. After releasing eS26 from its importins, Tsr2 would impose another regulatory level on eS26 by preventing its roles in pre-mRNA binding and splicing inhibition, thereby ensuring its incorporation into pre-90S subunits. Since eS26 might only be involved in mRNA interactions and splicing when its levels are misregulated, the C-terminus of Tsr2 might be more critical for Tsr2 function under such conditions. Therefore, it will be interesting to analyze whether the Tsr2 variant lacking its C-terminus, Tsr2<sup>1-152</sup>, is also able to rescue the slow growth of Tsr2-depleted cells under ribosomal stress.

In humans, balanced levels of eS26 are essential, since both increased and decreased levels trigger p53 activation and lead to cell cycle arrest, apoptosis or cellular senescence. Although no p53 homolog exists in yeast, it was shown that human p53 inhibits proliferation in S. cerevisiae (Nigro <em>et al.</em>, 1992), suggesting that a similar checkpoint might exist in yeast. This points to a possible regulatory role of Tsr2 in keeping eS26 ready for incorporation into pre-ribosomes and preventing the activation of a p53-like pathway.

2. A FAMILY OF ESCORTINS?
Adapted from Schütz <em>et al.</em>, 2014.

More than 20 years ago, a system was envisioned to efficiently transfer r-proteins from the NPCs towards the nucleolus (Russell and Tollervey, 1992). Here, we identify Tsr2 as the first component of this transfer system that connects the nuclear import machinery with the ribosome assembly pathway. We propose the term “escortin” to describe this “linker” function.

Aggregating r-proteins in the nucleolus aggravate the toxicity of a <em>C. elegans</em> Huntington disease model and decrease their lifespan (David <em>et al.</em>, 2000b).
2010), emphasizing the importance to safely transfer r-proteins to the assembling pre-ribosomes. Due to their unstable and aggregation-prone nature (Koplin et al., 2010) we envisage an escortin network to securely connect the nuclear import machinery with the ribosome assembly pathway. Intriguingly, like in the case of Kap123:eS26 complex, RanGTP is unable to efficiently release uL14 (human Rpl23a) from importin 7 (RanBP7) (Jäkel and Görlich, 1998). Moreover, we found that yeast r-proteins (uS14, eS31a and eS8a) bound to Kap123 were not released upon RanGTP treatment (Figure S8) suggesting that these r-proteins may require specific escortins for their release. Affinity purifications coupled to mass spectrometry have identified >200 non-ribosomal factors that are directly involved in ribosome assembly (Bassler et al., 2001; Dragon et al., 2002; Fatica et al., 2002; Grandi et al., 2002; Harnpicharnchai et al., 2001; Nissan et al., 2002; Schäfer et al., 2003). However, escortins, which are not stably bound to pre-ribosomal particles, may have escaped identification.

Individual subunits/sub-complexes of other macromolecular complexes involved in genome replication, genomic stability and gene expression must be imported into the nucleus prior to their assembly. The fate of these cargoes after being released from importins in the nucleus remains largely unexplored. Many of these components may rely on escortins that will ensure their transfer to their assembly site. Thus, we expect that the list of escortins for ribosome assembly and other biological pathways will expand in the near future. The identification of new escortins might give valuable insight into nuclear organization and regulation.

3. TSR2 AND eS26 IN DBA
Adapted from Schütz et al., 2014.

Similar to the Tsr2-depletion, both DBA mutants (eS26D33N and eS26C77W) accumulate 20S pre-rRNA in the cytoplasm (Figure 25C). The eS26C77W mutant interacted poorly with its import receptors, suggesting that the inability to interact with importins may cause its degradation. Notably, the eS26D33N mutant interacted with Kap123, Kap104, Pse1 and Tsr2 in vitro.
(Figure 26A and Figure 26B). We speculate that the *in vivo* instability of this variant might be due to a failure to incorporate eS26 into the 90S pre-ribosome.

Several mutations in eS26 have been linked to DBA, the majority of which are in the start codon and are thought to result in reduced levels of the r-protein eS26, thereby causing haploinsufficiency (Doherty *et al.*, 2010). Notably, eS26 levels are strongly reduced in Tsr2-depleted cells. Interestingly, about half of DBA cases are due to unidentified mutations. Based on these data, we speculate that the *TSR2* gene may be a potential hotspot for DBA.

A mutation within human Tsr2 (Tsr2E64G) has been recently linked to DBA (Gripp *et al.*, 2014). However, this residue is not conserved between humans and yeast. We mutated L84 in yeast Tsr2, which corresponds to the residue E64 mutated in DBA patients and introduced Tsr2L84G into yeast. Tsr2L84G rescued the slow growth of Tsr2-depleted cells (Figure S14), indicating that the protein variant is functional. Since human Tsr2 can partially rescue the slow growth of Tsr2-depleted cells, we analyzed the effect of human Tsr2E64G in yeast. Cells expressing Tsr2E64G displayed a slight growth defect (Figure S14), suggesting that this mutation somehow affects eS26 release, stability, transfer or incorporation into pre-ribosomes. Whether human Tsr2E64G is able to interact with eS26, and whether this variant can release eS26 from importins and stabilize it prior to its incorporation into pre-ribosomes remains to be investigated.

It is surprising that two mutations in the same gene and a mutation in Tsr2, which all might be expected to reduce eS26 levels, cause different physical anomalies in humans. The D33N mutation in eS26 causes a classical DBA phenotype, whereas mutation C77W in eS26 and E64G in Tsr2 cause DBA associated with Klippel-Feil and Treacher Collins syndromes, respectively (Cmejla *et al.*, 2011; Gripp *et al.*, 2014). It is speculated that r-proteins are involved in the recognition of specific mRNAs for translation. Since eS26 is involved in binding the 5′ UTR of mRNAs it is tempting to speculate that different mutations within eS26 might affect its ability to recognize specific mRNAs.

Mutations in Tsr2 might affect its own stability or the binding to eS26,
either of it resulting in reduced eS26 stability and incorporation. However, although the levels of eS26 might be reduced in Tsr2 mutant cells, the ability of residual eS26 to recognize specific mRNAs might remain unchanged. Could the eS26 levels per se somehow affect the DBA associated phenotypes? eS26C77W, which causes a more severe phenotype in DBA patients than eS26D33N, appears to be less stable in yeast than eS26D33N. Whether this is also the case in humans needs to be tested. Human Tsr2 was recently suggested to be involved in the inhibition of the transcriptional activity of NF-κB, thereby inducing apoptosis (He et al., 2011). Whether Tsr2 has a role independent of eS26 in the NF-κB signaling pathway and whether this role links Tsr2 to DBA associated with TCS remains unknown.

4. **FURTHER WORK ON TSR2 AND eS26**

Although we have demonstrated that Tsr2 releases eS26 in a RanGTP-independent manner from its transport receptor, the molecular mechanisms of this process as well as downstream events are not yet understood. How does Tsr2 release eS26 from its importins? Do structural rearrangements in eS26 or Tsr2 contribute to importin release and 90S binding? Does Tsr2 directly contribute to the incorporation of eS26 into the pre-ribosome or which other factors are involved in this process? Solving the structure of eS26 bound to Tsr2 and to an importin will help to answer these questions. Because of its large size, the structure of eS26 bound to an importin is likely to require X-ray crystallography. However, we are currently using NMR spectroscopy to solve the structure of the approximately 40 kDa Tsr2:eS26 complex. This structure will provide insight into the function and mechanism of escortins as well as the role of the acidic C-terminus of Tsr2.

5. **IMPLICATIONS OF THIS STUDY**

This work presents the discovery of the conserved carrier Tsr2, which coordinates the safe transfer of the eukaryote specific r-protein eS26 to 90S pre-ribosomes. In addition to uncovering a new RanGTP-independent mechanism that terminates the nuclear import process of the r-protein eS26
and promotes its incorporation into pre-ribosomes, we believe that this work will stimulate research on transport processes of diverse cargoes and especially how they are coupled to downstream assembly/signaling events. Escortins might not be restricted to ribosomal assembly, but may also be required for the assembly of other macromolecular complexes, including RNA polymerases and complexes required for DNA metabolism. Thus, we expect that the list of escortins for both ribosomal proteins and other biological pathways to expand in the near future. Since escortins are not ribosome-bound assembly factors and might therefore be difficult to identify, the structure of Tsr2 could tremendously help to identify further escortins with similar folds based on structural comparisons.

In addition to contributing to our understanding of intracellular transport, this work also sheds light on the molecular mechanisms underlying ribosomopathies. RPS26 is one of the most frequently mutated genes causing DBA. eS26 mutants associated with DBA phenocopy Tsr2-depleted cells, i.e. they show strongly reduced eS26 protein levels and accumulate aberrant pre-40S subunits containing 20S pre-rRNA in the cytoplasm. Interestingly, the mutations causing DBA in about 50% of cases remain unknown. Our data identifies the TSR2 gene as a potential hotspot for DBA, and provides a mechanistic framework towards understanding eS26-associated DBA.
MATERIALS AND METHODS
Parts of materials and methods were adapted from Altvater et al., 2014

1. GENETIC METHODS

1.1. Yeast strains

Preparation of media and genetic manipulations were performed according to established procedures. Plasmids used in this study are listed in Table S2. Details of plasmid construction will be provided upon request. All recombinant DNA techniques were performed according to established procedures using *E. coli* XL1 blue cells for cloning and plasmid propagation. All cloned DNA fragments and mutagenized plasmids were verified by sequencing.

All *Saccharomyces cerevisiae* strains used in this study are listed in Table S3. Genomic disruptions, C-terminal tagging and promoter switches at genomic loci were performed by homologous recombination. DNA fragments for homologous recombination were generated by polymerase chain reaction (PCR) using pFA and pYM plasmids as templates and S1, S2, S3, S4 or F1, F2, R1, R2, R4 primer (Janke et al., 2004; Longtine et al., 1998; Puig et al., 2001).

1.2. Mating and sporulation of yeast strains

For mating, MATa and MATα cells were mixed in equal amount on a YPD plate and incubated over night at 30°C. Diploid cells were selected on double selective plates and grown for 1-2 days at 30°C. After selection, diploids were transferred to a plate containing sporulation agar (Formedium, Norfolk, UK) and incubated for 5-7 days at 22°C.

1.3. Spore digestion and microdissection

A small amount of cells was taken from the sporulation plates and resuspended in 50 µl dH2O. 5 µl zymolyase 20T (final concentration of 0.3 mg/ml; Seigaku Corporation, Tokyo, Japan) was added. After an
incubation of 6-10 min at RT, 400 µl H₂O was added. 25 µl of this mixture was
spread on the boarder of a YPD plate. After drying, the digested tetrads were
microdissected into single haploid spores using a MSM-30 microdissection
device (Singer instruments, Somerset, UK).

2. CELL BIOLOGICAL METHODS

2.1. Fluorescence microscopy of live cells

Pre-40S subunit export, monitored by localization of uS5-GFP and
localization of GFP-eS26 was performed as previously described (Altvater et
al., 2014; Faza et al., 2012). Cells were visualized using DM6000B
microscope (Leica, Wetzlar, Germany) equipped with HCX PL Fluotar
63×/1.25 NA oil immersion objective (Leica). Images were acquired with a
fitted digital camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan)
and Openlab software (Perkin-Elmer, Waltham, MA, USA).

2.2. Indirect immunofluorescence

For indirect immunofluorescence the Tsr2-TAP strain was grown at
30°C to OD₆₀₀ 0.4-0.8. Cells were harvested at 2,000 rpm and resuspended in
1 ml culturing media. After addition of 120 µl 37 % Formaldehyde (Sigma-
Aldrich, St. Louis, MO, USA), cells were fixed for 1 h at 30°C at 1,000 rpm.
After fixation, cells were harvested and washed in 1 ml PBS/Sorbitol (1x PBS,
20 % (w/v) Sorbitol). Cells were digested in 1 ml PBS/Sorbitol containing 50 µl
Zymolyase 100T (5 mg/ml, Seigaku Corporation) for 30 min at 30°C on a
slowly rotating wheel. After digestion, cells were harvested at 3,300 rpm for
3 min, washed in 1 ml PBS/Sorbitol and resuspended in 20-100 µl
PBS/Sorbitol (accordingly to the pellet size). 20 µl of poly-L-lysine (0.1% (w/v),
Sigma-Aldrich) was applied per well, incubated for 5 min, washed three times
with dH₂O, aspirated and air-dried. 25 µl cells were then added onto poly-L-
lysine-coated slide well (8 well, 6 mm, Menzel-Gläser Diagnostika,
Braunschweig, Germany). Non-adhering cells were removed after 30 s and
the slide was transferred into an ice-cold methanol bath for 6 min. After an
incubation of 30 s in an ice-cold acetone bath, microscopy slides were dried. Blocking was performed in 30 µl BSA/PBS (1x PBS, 0.2 % w/v BSA) for 1 h in the dark at RT. After removal of the blocking solution 25 µl of affinity-purified polyclonal antibodies against the TAP-tag (1:1,000; Thermo Scientific, Rockford, IL, USA) in BSA/PBS was added to the cells and incubated over night at RT in the dark. The cells were washed three times with BSA/PBS and incubated for 5 h with 20 µl of AlexaFluor568 coupled anti-rabbit (Molecular Probes, Inc., Eugene, OR, USA) at RT in the dark. After washing with BSA/PBS, the cells they were incubated for 30 s with DAPI (1 µg/ml in BSA/PBS; Sigma-Aldrich) to stain nuclear and mitochondrial DNA. After DAPI staining, cells were washed three times with BSA/PBS, slides were dried and mounted with Mowiol (Calbiochem, San Diego, CA, USA).

2.3. Fluorescence in-situ hybridization

Yeast cells were grown in appropriate temperature and media to mid-log phase. For fixation, 5.4 ml of 37% formaldehyde (RNase-free, Sigma-Aldrich) (final concentration of 4% (v/v)) was added to 44.6 ml of cell culture. Then, cells were gently shaken for 15 min at appropriate temperature (i.e., the temperature at which cells were grown). Cells were centrifuged for 1 min at 3,300 rpm at RT. Cells were resuspended in 4% formaldehyde (RNase-free) in 0.1 M KPO$_4$ pH 6.4, and fixed at RT for 2 h 45 min. Then cells were washed twice with 10 ml 0.1 M KPO$_4$ pH 6.4, centrifuged (3,300 rpm for 1 min), resuspended in 1 ml wash buffer and transferred into an Eppendorf tube. Then cells were pelleted at 6,000 rpm for 30 s, resuspended in 1 ml wash buffer (100 mM KPO$_4$ pH 6.4, 1.2 M Sorbitol) containing 0.5 mg/ml Zymolyase 100T (Seigaku Corporation, Tokyo, Japan) and 10 mM Ribonucleoside Vanadyl Complex (RVC, Sigma-Aldrich) and incubated at 30°C for 30 min on a rotating wheel. Cells were pelleted at 3,000 rpm for 3 min, washed once with 1 ml wash buffer, and resuspend in wash buffer (ca. 2 vol. of pellet). 20 µl of poly-L-lysine (0.1% (w/v), Sigma-Aldrich) was applied per well, incubated for 5 min, aspirated and air-dried. Then 20 µl of cells were applied onto the poly-L-lysine-coated slide well (8 well, 6 mm, Menzel-Gläser Diagnostika). After 5 min non-adhering cells were removed, wells were washed with 100 µl
2x SSC (300 mM NaCl, 30 mM Sodium citrate) and then incubated for 10 min in 100 µl 2x SSC. 12 µl pre-hybridization buffer (50% Formamide (Applichem, Darmstadt, Germany), 10% Dextran sulphate (Calbiochem), 125 µg/ml E. coli tRNA (Fluka, St. Louis, MO, USA), 500 µg/ml Herring sperm DNA (Sigma-Aldrich), 0.02% Denhardt’s Solution (Sigma-Aldrich), 1 mM dithiothreitol (DTT), 600 mM NaCl, 60 mM Sodium citrate) was applied onto the wells, and cells were incubated at 37°C for 1 h in a humid chamber. Then 1 µl of 5 pmol/µl Cy3-labeled ITS1 oligonucleotide probe (5’-Cy3-ATG CTC TTG CCA AAA CAA AAA AAT CCA TTT TCA AAA TTA AAT TTC TT-3’, Thermo-Scientific), complementary to the 5’ portion of ITS1 (Altvater et al., 2014; Faza et al., 2012; Jakovljevic et al., 2004), was applied. Cells were incubated overnight in a humid chamber at 37°C. Slides were washed in a staining jar with 100 ml 2x SSC buffer, and incubated for 5 min at RT in 100 ml 2x SSC. Nuclear and mitochondrial DNA was stained in 100 ml 1x SSC (150 mM NaCl, 15 mM Sodium citrate) containing 5 µg/ml DAPI (Sigma-Aldrich) for 5 min. Then cells were washed in 100 ml 0.5x SSC (75 mM NaCl, 7.5 mM Sodium citrate) for 30 min at RT. Slides were air-dried, mounted with Mowiol (Calbiochem) and dried for 1 h at RT.

3. BIOCHEMICAL METHODS

3.1. Tandem affinity purifications

Yeast cell cultures were grown to OD$_{600}$ 3-3.5, and harvested at 5,000 rpm for 15 min at 4 °C. Then cells were washed with dH$_2$O, and pelleted again at 5,000 rpm for 5 min at 4°C. The pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 7.2, 1.5 mM MgCl$_2$, 0.15 v/v Igepal CA-630 (Sigma-Aldrich), 100 mM NaCl (for pre-40S particles), 50 mM NaCl (for Tsr2-TAP purification) or as indicated) containing complete protease inhibitor, 1 mM PMSF (Axon Lab AG, Baden-Dättwil, Switzerland) and 1 mM DTT in a final volume of 25 ml. Cells were lysed at 500 rpm for 20 min with beads in a Pulverisette 6 planetary mill (Fritsch, Idar-Oberstein, Germany) at 4°C. The lysates were clarified by centrifugation first at 5,000 rpm for 10 min at 4°C, subsequently at 18,000 rpm for 30 min at 4°C. 150 µl of equilibrated IgG
Sepharose (GE Healthcare, Buckinghamshire, UK) was added to the cleared cell lysate. After 1 h incubation at 4°C on a rotating wheel, beads were washed with 10 ml lysis buffer containing 0.5 mM DTT. TEV protease cleavage was performed in 5 ml lysis buffer containing 0.5 mM DTT and 10 µl TEV protease (1 mg/ml) for 2 h at 16°C to release bound particles from the IgG Sepharose. The TEV-eluate was collected and the IgG Sepharose beads washed with 4 ml lysis buffer containing 0.5 mM DTT. To the TEV-eluates CaCl₂ and DTT (to final concentrations of 0.5 mM and 1 mM, respectively) and 150 µl Calmodulin Sepharose (GE Healthcare) were added and incubated for 1 h at 4°C on a rotating wheel. The beads were washed with 15 ml lysis buffer containing 2 mM CaCl₂ and 1 mM DTT. Bound particles were eluted at 35°C four times for 10 min in 300 µl elution buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 5 mM EGTA, final elution volume: 1.2 ml). The proteins in the Calmodulin eluates were precipitated by adding 10% (v/v) trichloroacetic acid (TCA) and incubated on ice for 15 min. Precipitated proteins were pelleted at 14,000 rpm at 4°C for 10 min, washed with 1 ml cold acetone and pelleted again. The pellet was air-dried and resuspended in 20-50 µl 1x LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Samples were heated for 10 min at 70°C, separated on NuPAGE 4-12% Bis-Tris gradient gels (Invitrogen) and subjected to silver staining or Western analyses.

To analyze particles for SRM-MS, proteins in the TEV- or Calmodulin eluates were precipitated in 10% TCA and resuspended in denaturing buffer (8 M urea, 50 mM NH₄HCO₃, and 5 mM EDTA). Then the proteins were reduced with 12 mM DTT for 30 min at 32°C and alkylated with 40 mM iodoacetamide for 45 min at 25°C. The samples were diluted 1:5 with 0.1 M ammonium bicarbonate and digested overnight with sequencing-grade porcine trypsin (Promega, Madison, WI, USA) at an enzyme/substrate ratio of 1:100. The digestion was stopped with formic acid to a final concentration of 2%. The peptide mixtures were then desalted on Sep-Pak C18 cartridges (Waters, Dättwil, Switzerland), eluted with 80% acetonitrile, dried by vacuum centrifugation, and resuspended in 0.15% formic acid (Altvater et al., 2012).

To analyze RNAs after TAP purification, 1 ml Phenol-Chlorofom-Isoamylalcohol (Thermo Scientific) was added to 1 ml Calmodulin-eluates.
Samples were vortexed for 1 min and centrifuged at 14,000 rpm for 3 min. The aqueous phase was transferred to a fresh tube, 100 µl 3 M NaOAc pH 5.3, 1 µl Glycoblu (Ambion, Austin, TX, USA), and 800 µl 100 % Isopropanol were added, vortexed and centrifuged at 4 °C at 14,000 rpm for 30 min. The pellet was washed with ice-cold 80 % Ethanol. After centrifugation at 4 °C at 14,000 rpm for 10 min and air-drying the pellet, it was resuspended in 20 µl dH₂O. 1 µg of total RNA was separated on a 1.2% Agarose/formaldehyde gel for 1.5 h at 200 V.

3.2. Purification of recombinantly expressed proteins

All recombinant proteins were expressed in *E. coli* BL21 cells containing the pRARE plasmid, which expresses tRNAs for codons rarely used in *E. coli*. Expression was induced with 1 mM IPTG (AppliChem) and proteins were expressed at 4°C over night. For the expression of recombinant eS26 (GST-eS26, eS26, eS26-FLAG), 100 µM ZnCl₂ was added to the expression media. Prior cell lysis, 1 mM DTT, 1mM PMSF and ½ tablet of complete protease inhibitor cocktail (Roche, Basel, Switzerland) was added to the cells. Cell lysis was performed by sonication four times for 1 min at 4°C by a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). His₆-tagged proteins were lysed and affinity purified in 50 mM Hepes pH 7.5, 50 mM NaCl, 10% glycerol, 1 mM DTT using Ni-NTA Agarose (GE Healthcare), GST-fusion proteins were purified in PBSKMT (150 mM NaCl, 25 mM sodium phosphate, 3 mM KCl, 1 mM MgCl₂, 0.1% Tween, pH 7.3) using Glutathione Sepharose (GE Healthcare). His₆-tagged proteins were eluted from Ni-NTA Agarose by 150 mM Imidazole in 50 mM Hepes pH 7.5, 50 mM NaCl, 10% glycerol, 1 mM DTT. GST-tagged proteins were eluted in GSH-elution buffer (50 mM Tris-HCl pH 8, 3.2 mg/ml reduced L-Glutathione (Sigma-Aldrich), 150 mM NaCl). GST-tagged importins, His₆-tagged importins and RanGTP (His₆-Gsp1Q71L-GTP) were expressed and purified as previously described (Fries *et al.*, 2007; Maurer *et al.*, 2001; Solsbacher *et al.*, 1998).
3.3. In vitro RNA transcription and RNA purification

RNA transcription of the 3’ end of the 18S rRNA was performed in a final volume of 10 ml using 50 µM of the 40 bp ssDNA template (5’-TAA TGC ATC TCC AGG TTC CTA TAG TGA GTC GTA TTA-3’, Microsynth, Balgach, Switzerland) containing the T7 promotor region, 50 µM T7 promotor primer (5’-TAA TAC GAC TCA CTA TAG-3’, Microsynth), 38.6 mM MgCl₂, 5 mM of each dNTP, 1 µM T7 RNAP in transcription buffer (40 mM Tris-HCl pH 8.0, 1 mM spermidine, 0.01% Triton X-100 und 5 mM DTT). The reaction mixture was incubated for 3 h at 37°C. To stop the reaction, 50 mM EDTA were added and the reaction mixture was filtrated through a 0.2 µM pore size filter. Then the RNA was purified by anion-exchange chromatography on a preparative Dionex DNAPac PA-100 column (Thermo Scientific) at 85°C equilibrated in buffer A (12.5 mM Tris-HCl at pH 8.0, 6 M urea). The RNA was eluted in a linear gradient from 30-75% buffer B (12.5 mM Tris-HCl at pH 8.0, 0.5 M NaClO₄, 6 M urea) and fractions containing the purified RNA were determined by 16% urea acrylamide gels. The RNA was subsequently precipitated three times by butanol extraction (Cathala and Brunel, 1990) and the obtained RNA pellet was dissolved in water and freeze-dried to remove residual butanol. The lyophilized RNA was then dissolved in the desired buffer.

3.4. Binding assays with recombinant proteins

Recombinant GST-Tsr2 was immobilized in PBSKMT on Glutathione Sepharose, and incubated with E. coli lysates containing recombinant eS26, eS26^{FLAG}, eS26D33N^{FLAG}, eS26C77W^{FLAG} for 1 h at 4°C. After incubation, the immobilized GST-proteins were washed three times with 1 ml PBSKMT at 4°C. The bound proteins were eluted with 2x LDS. The in vitro binding studies between recombinant eS26^{FLAG}, eS26D33N^{FLAG}, eS26C77W^{FLAG}, Tsr2, Tsr2:eS26 complex and yeast importins as GST-fusion proteins were performed as previously described (Solsbacher et al., 1998). 1/5th of the bound proteins and input (eS26, eS26^{FLAG}, eS26D33N^{FLAG}, eS26C77W^{FLAG}) were analyzed on a Coomassie Blue stained gel. 1/10th of the bound proteins...
and 1/1000\textsuperscript{th} of the input was used for Western analyses.

To dissociate the GST-importin:eS26\textsuperscript{FLAG} (Kap123, Pse1 and Kap104) complex or GST-Kap123:eS31\textsuperscript{FLAG}, GST-Kap123:eS8\textsuperscript{FLAG}, GST-Kap123:eS14\textsuperscript{FLAG} complexes pre-immobilized GST-importin:τ-protein complexes were incubated with buffer alone or 3 nM of 3' end of 18S rRNA (only for eS26\textsuperscript{FLAG}), 1.5 µM Tsr2, 1.5 µM His\textsubscript{6}-Tsr2 (only eS26\textsuperscript{FLAG}) and/or 1.5 µM RanGTP (His\textsubscript{6}-Gsp1Q71L-GTP) for 1 h at 4°C (protocol modified from Rothenbusch et al., 2012). To show that eS26 stably associated with Tsr2 after release from importins, the supernatant of the samples with buffer alone and His\textsubscript{6}-Tsr2 were incubated with Ni-NTA Agarose (GE Healthcare) for 1 h at 4°C. For dissociation kinetics, 1.5 µM RanGTP (His\textsubscript{6}-Gsp1Q71L-GTP) or Tsr2 were added to pre-immobilized importin:eS26\textsuperscript{FLAG} complexes and samples were withdrawn at 1, 2, 4 and 8 min. Bound proteins were eluted in 2x LDS/SDS-sample buffer by incubating at 70-95°C and separated by SDS-PAGE. Proteins were visualized by Coomassie Blue staining or by Western analyses using antibodies against Tsr2 and eS26.

Recombinant GST-Tsr2\textsuperscript{153-205} was immobilized in PBSKMT on Glutathione Sepharose, washed three times with 1 ml PBSKMT and incubated with \textit{E. coli} lysates containing recombinant eS26\textsuperscript{FLAG}, His\textsubscript{6}-Tsr2 eS26\textsuperscript{FLAG} complex, His\textsubscript{6}-Tsr2\textsuperscript{1-152} eS26\textsuperscript{FLAG} complex for 1 h at 4°C. Then, 20 µg/ml (final concentration) RNaseA (Thermo Scientific) was added and incubated for 1 h at 4°C. Beads with bound proteins were washed three times with PBSKMT, resuspended in PBSKMT containing 10 mM RVC. 20 µM 3’ end of the 18S rRNA was added and incubated for 1 h at 4°C. After incubation, the immobilized GST-proteins were washed three times with PBSKMT at 4°C. The bound proteins were eluted with LDS.

To test whether Tsr2 or Tsr2\textsuperscript{1-152} can release RNA from GST-eS26, GST-eS26 was immobilized in PBSKMT on Glutathione Sepharose (GE healthcare), and incubated with increasing amounts of purified Tsr2 or Tsr2\textsuperscript{1-152} (0.3-14 µM as indicated in figure legend). The flow-through after Tsr2 binding was collected. Bound proteins were eluted by GSH-elution buffer (50 mM Tris-HCl pH 8, 3.2 mg/ml reduced L-Glutathione (Sigma-Aldrich), 150 mM NaCl). Absorption at 260 nm of flow-through and bound fractions was
determined on a NanoDrop 1000 spectrophotometer (Thermo Scientific). The sample was then mixed with RNA loading dye and analyzed on a denaturing RNase free ethidiumbromide stained Agarose gel (1.2% (w/v) Agarose (Invitrogen), 0.7% Formaldehyde, 0.1 µg/mL Ethidiumbromide (Themo Scientific) in 1x FA buffer (50 mM Heps-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA).

To analyze whether importins can release eS26 from Tsr2 co-expressed GST-Tsr2:eS26 was immobilized in PBSKMT on Glutathione Sepharose and incubated with increasing amounts of *E. coli* lysates containing recombinant His6-Kap123 (50 nM-800 nM) at 4°C for 1 h. After incubation, the immobilized proteins were washed three times with PBSKMT and eluted with 2x LDS.

### 3.5. SDS-PAGE

Whole cell extract or affinity purified proteins, stored at -20°C, were heated for 5 min to 70°C and centrifuged at 14,000 rpm. 1-10 µl of the supernatant was loaded on SDS-polyacrylamide gels or onto NuPAGE Novex 4-12% gradient gels (Invitrogen). For SDS-polyacrylamide gels, proteins were separated at 150 V for 55 min and visualized by Western blotting. For gradient gels, electrophoresis was performed in XCell SureLock Mini-Cells (Invitrogen) at 150 V for 1 h 15 min in MOPS buffer (50 mM MOPS pH 7.7, 50 mM Tris-Base, 0.1% SDS, 1mM EDTA). Proteins were visualized by Western analyses or silver staining.

### 3.6. Silver staining

After electrophoresis, NuPAGE Novex 4-12% gradient gels were washed with dH2O and then fixed for 1 h in 100 ml of 30% ethanol, 15% acetic acid. Gels were washed in dH2O and incubated over night in 100 ml 0.5 M sodium acetate, 25% ethanol, 12 mM Na2S2O3 (Sigma-Aldrich), 0.125% glutaraldehyde (Sigma-Aldrich). Gels were washed three times for 10 min in dH2O and stained for 30 min in 100 ml 0.1% AgNO3 (Sigma-Aldrich), 0.011%
formaldehyde. Gels were briefly rinsed in dH₂O and 100 ml developer solution (0.235 M Na₂CO₃, 0.011% formaldehyde) was added. Reaction was stopped by addition of 10 ml 0.5 M EDTA. Subsequently, gels were washed three times in dH₂O. Gels were digitalized using a Perfection V500 Photo scanner (Epson, Suwa, Japan).

### 3.7. Western blotting

Following electrophoresis, acrylamide gels or NuPAGE Novex 4-12% gradient gels were shortly incubated in blotting buffer (25 mM Tris-Base, 192 mM Glycine). Proteins were then transferred onto nitrocellulose membranes (GE Healthcare) at 120 mA for 45 min using a Trans-Blot SD Semi-Dry Elecrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). Membranes were blocked for 1 h at RT in 3% (w/v) milkpowder in PBST (1x PBS, 0.05% Tween20) (hereafter termed 3% milk). Primary antibody was diluted in 1% (w/v) milkpowder in PBST and membranes were incubated in primary antibody either for 1-2 h at RT or over night at 4°C. Membranes were washed three times in PBST, then secondary antibody coupled to horseradish-peroxidase (HRP) (Sigma-Aldrich) diluted in 1% milk was added to the membrane and incubated for 1-2 h at RT. Membranes were washed three times in PBST. For detection, Clarity Western ECL Substrate (Bio-Rad) chemiluminescence reagents were applied onto the membrane, the signal was captured using Fuji Super RX X-ray films (Fujifilm, Tokyo, Japan) and developed in a SRX-101A (Konika, Tokyo, Japan). Films were digitalized using a Perfection V500 Photo scanner (Epson). Antibodies used in this study are listed in Table S4.

### 3.8. Sucrose gradient analysis

Sedimentation analysis of yeast lysates by sucrose gradient ultracentrifugation was performed as described previously (Altvater et al., 2014; Kemmler et al., 2009). Yeast cells were grown in appropriate medium and temperature to mid-log phase. To preserve polysomes 100 µg/ml cycloheximide (AppliChem) was added to the cultures and incubated on ice.
for 5 min. Cells were then harvested at 4,000 rpm for 5 min at 4°C and washed in 20 ml lysis buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 30 mM MgCl₂, 1 mM DTT, 100 µg/ml cycloheximide). Cells were resuspended in 1 ml lysis buffer and quickly pelleted (14,000 rpm for 5 s). Then cells were resuspended in 300-500 µl lysis buffer with 0.2 g glass beads (400-600 µm diameter) and lysed by vortexing (Disruptor Genie, Scientific Industries, Bohemia, NY, USA) at maximum speed for 8 min at 4°C. The lysate was recovered and clarified by centrifugation at 14,000 rpm for 10 min at 4°C. To the cleared supernatant 5% of glycerol was added and aliquots of 4 units of A₂₆₀ were made.

The cell lysates were layered on top of a sucrose density gradient (50 mM Tris-HCl pH 7.5, 50 mM NH₄Cl, 12 mM MgCl₂, 1 mM DTT, 7-50% (w/v) sucrose) in a SW41 Polyallomer tube (Beckman Coulter, Pasadena, CA, USA). The sucrose gradient was subjected to ultracentrifugation at 39,000 rpm for 3 h at 4°C using a Beckmann SW41 swing bucket rotor. Polysome profiles were recorded at 254 nm and gradients were fractionated (500 µl fractions) using a Teledyne Isco density gradient recording system (Teledyne Isco, Lincoln, NE, USA).

For Western analyses, 500 µl fractions were precipitated by TCA, washed in acetone, resuspended in 50 µl 1x SDS sample buffer and separated by SDS-PAGE. Tsr2, eS26 and uL3 were detected by Western analyses. For rRNA analysis, 500 µl fractions were collected and diluted with an equal volume of lysis buffer. RNA was extracted with Phenol-Chlorofom-Isoamylalcohol and precipitated in isopropanol. RNA pellets were washed with 80% ethanol and resuspended in 20 µl dH₂O. rRNAs were then separated on a 1.2% Agarose/formaldehyde gel for 1.5 h at 200 V. For Northern analysis, rRNAs were blotted onto a Hybond-XL (GE Healthcare) membrane by capillary transfer and probed for 18S rRNA (5'-CAT GCA TGG CTG AAT CTT TGA GAC), 20S pre-rRNA (5'-GGT TTT AAT TGT CCT ATA ACA AAA GC) and 25S rRNA (5'-TGC CGC TTC ACT CGC CGT TAC) using radioactively labeled probes. rRNAs were detected using phosphoimaging screens (GE Healthcare).
3.9. Aggregation assay

The aggregation assay in presence of PreScission protease was performed in a 384-well plate (Polystyrene, clear bottom, low volume, Corning, USA). In each well 33 µM GST-eS26 and a given concentration of Tsr2 (0-266 µM) in PBSKMT was pre-incubated for 1 h at 4°C (final volume: 90 µl). 250 nM of PreScission protease was added to initiate aggregation. Aggregation of free eS26 was measured at 450 nm using a Multiskan GO plate reader (Thermo Scientific, USA). As controls, scattering intensities of individual components used in the aggregation assay such as 33 µM of GST-eS26 alone, 266 µM of Tsr2 alone, PreScission protease and buffer were measured. Four replicates were performed for each sample.

The aggregation assays in presence of RNaseA and Tsr2 fragments were performed in a 96-well plate (Polystyrene, clear bottom, Sarstedt, Nümbrecht, Germany). In each well ~40 µM GST-eS26 and a given concentration of Tsr2, Tsr2\(^{1-152}\), Tsr2\(^{153-205}\) or a combination of Tsr2\(^{1-152}\) and Tsr2\(^{153-205}\) (0-160 µM) in PBSKMT was pre-incubated for 1 h at 4°C (final volume: 60 µl). Then RNaseA (Thermo Scientific) in a final concentration of 17 µg/ml was added to initiate aggregation. Aggregation of free eS26 was measured at 450 nm using a SpectraMAX Plus (Molecular Devices, Sunnyvale, CA, USA) plate reader. As controls, scattering intensities of individual components used in the aggregation assay such as 40 µM of GST-eS26 alone, 160 µM of Tsr2, Tsr2\(^{1-152}\), Tsr2\(^{153-205}\) alone, RNaseA and buffer were measured. Two replicates were performed for each sample.

3.10. In vitro degradation assay

*E. coli* lysate containing recombinant eS26 was incubated with either buffer alone or with 75 µM Tsr2 or 400 µl *E. coli* lysate containing GST-Kap123 at 37°C at 1,000 rpm in 1 ml native yeast lysate. 150 µl of the reaction mixture were withdrawn at the indicated time points. Aggregated proteins were removed by centrifugation for 8 min at 14,000 rpm. Proteins in supernatant were precipitated by 10% TCA and pelleted for 8 min at
14,000 rpm. Pellets were washed with 1 ml acetone and pelleted again. Air-dried pellets were resuspended in 100 µl LDS and boiled for 10 min at 70°C.

### 3.11. Selective reaction monitoring mass-spectrometry

#### 3.11.1. SRM assay development, quantitation and statistical analysis

##### 3.11.1.1. Sample preparation

Affinity purified pre-40S particles were processed for mass spectrometric analysis as described earlier (see Tandem affinity purifications; Altvater et al., 2012). Affinity-purified protein samples were denatured and cysteine residues were reduced and alkylated. After tryptic digestion the peptides were purified with C18 columns. Before mass spectrometric analysis, 11 retention time calibration peptides (iRT peptides, RT-kit WR, Biognosys) were added to every sample at a ratio of 1:20.

##### 3.11.1.2. SRM assay development

To develop SRM assays, peptide samples of the affinity purified pre-40S particles were analyzed on a nanoLC 1Dplus system (Eksigent) connected to a TripleTOF 5600+ mass spectrometer (ABSciex). Peptides were separated by reversed-phase liquid chromatography on a 20 cm fused silica microcapillary (75 µm inner diameter, New Objective) packed in-house with 3 µm C18 beads (Magic C18 AQ, 200 Å pore size, Michrom BioResources, Auburn, CA, USA) with a linear gradient from 98% solvent A (98% acetonitrile, 0.1% formic) and 2% solvent B (98% acetonitrile, 0.1% formic acid) to 35% solvent B over 120 min at a flow rate of 300 nl/min. The mass spectrometer was operated in information-dependent acquisition (IDA) mode. MS1 spectra were recorded in the range of 360-1,460 m/z for 500 ms. Up to 20 precursor ions with charge state 2-5 were selected for fragmentation and MS2 spectra were recorded in the range of 50-2,000 m/z for 150 ms in high sensitivity mode. Selected precursor ions were excluded for 20 s after 1 occurrence. Raw data files were centroided and converted to mzML format.
using the ABSciex Data Converter and then converted to mzXML format using ProteoWizard MSConvert (Kessner et al., 2008).

MS2 spectra were searched with Sorcerer-SEQUEST (SageN Research) against a S. cerevisiae protein database (SGD, May 2013) to which the sequences of the 11 spiked-in iRT peptides and various common contaminants were added. Reversed sequences of all proteins were appended to the protein database to assess the number of false positive peptide-spectrum matches (Elias and Gygi, 2007).

Tryptic cleavage was defined to occur after lysine and arginine, unless followed by a proline residue, and peptides were allowed to have up to 1 non-tryptic end and up to 2 missed cleavages. Cysteine carbamidomethylation was added as static modification and methionine oxidation as variable modification. Precursor mass tolerance was set to 50 ppm. Resulting peptide-spectrum matches were statistically assessed using PeptideProphet and iProphet as part of the TPP (Deutsch et al., 2010; Keller et al., 2002; Shteynberg et al., 2011). The iProphet output was processed with MAYU (Reiter et al., 2009), which has been modified to work with iProphet probabilities. Peptide-spectrum matches were selected at a false discovery rate (FDR) of 0.07% to obtain a protein FDR of 1%. An in-house written script was used to convert all retention times into iRT values (Escher et al., 2012). SpectraST (Lam et al., 2008) was used to generate a consensus spectral library from which the 6 most intense fragment ions (b- or y-ions) per peptide precursor were selected in Skyline (MacLean et al., 2010). The final SRM assays for target proteins and iRT peptides are given in Table S5.

3.11.1.3. SRM analysis

The SRM data was acquired on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA) coupled to a nanoLC 1Dplus system (Eksigent). Peptides were separated by reversed-phase liquid chromatography on a 10.5-cm fused silica microcapillary (75 µm inner diameter, New Objective) packed in-house with 5 µm C18 beads (Magic C18 AQ, 200 Å pore size, Michrom BioResources) with a linear gradient from
95% solvent A (98% acetonitrile, 0.1% formic) and 5% solvent B (98% acetonitrile, 0.1% formic acid) to 35% solvent B over 35 min at a flow rate of 300 nl/min. The mass spectrometer was operated in positive mode using electrospray ionisation with a voltage of 1400 V. The capillary temperature was set to 280°C and the collision gas pressure to 1.5 mTorr. All transitions were monitored in scheduled mode with a retention time window of ±600 s, a cycle time of 2 s, and a mass window of 0.7 of half-maximum peak width (unit resolution) in Q1 and Q3.

The SRM data was analyzed manually in Skyline (MacLean et al., 2010). After removing non-detectable peptides and interfered transitions, peptide intensities (sum of integrated transition peak area) were exported for further processing in Excel. All peptides were normalized on the protein abundance of uS7 (Rps5). Peptides of each protein were ranked by their average intensity over all samples and the 3 most intense peptides were averaged to obtain an abundance value for every protein. The standard deviation was calculated assuming that the values are a sample of the entire population.

The SRM data can be viewed in and downloaded from Panorama: https://daily.panoramaweb.org/labkey/project/Aebersold/schubert/2014_Schuetz_Ribo-40S/begin.view.

4. MOLECULAR BIOLOGICAL METHODS

4.1. Polymerase chain reaction

Polymerase chain reactions (PCRs) were accomplished in a final reaction volume of 100 µl in the presence of 1-100 ng of template DNA, 0.2 mM of each dNTP (Fermentas), 0.5 µM forward and reverse primers, respectively, iProof reaction buffer (HF or GC buffer) and 2 U of iProof DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). PCR was performed in a Mastercycler PCR thermal cycler (Eppendorf, Hamburg, Germany) using the following conditions:
<table>
<thead>
<tr>
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<tbody>
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<tr>
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<td>30 sec</td>
</tr>
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<table>
<thead>
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</tr>
<tr>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

To verify that genomic integrations (deletions, truncations or promoter switch) occurred at the correct locus, clones grown after transformation were analyzed by colony PCR. Cells were resuspended in 100 µl NTES buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM Na$_2$EDTA) with 0.2 g glass beads (400-600 µm diameter) and lysed by vortexing (Disruptor Genie, Scientific Industries) at maximum speed for 8 min at 4°C. The lysate was centrifuged for 1 min at 14,000 rpm and 1 µl of supernatant used as template for PCR. pTEF$_{up}$, a primer annealing to the integrated fragment and a primer aligning upstream of the start codon of the gene of interest were used. PCR was performed in a final reaction volume of 25 µl in presence of 0.2 mM of each dNTP, 1x ThermoPol reaction buffer (200 mM Tris-HCl pH 8.8, 100 mM (NH$_4$)$_2$SO$_4$, 100 mM KCl, 20 mM MgSO$_4$, 1% Triton X-100) and Taq polymerase. The PCR was performed in a Mastercycler using the following conditions:

<table>
<thead>
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<th>Temperature</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
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<td>1-2 min</td>
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<tr>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>

PCR reactions to introduce point mutations into plasmids were performed in a reaction volume of 25 µl in presence of 30 ng plasmid as template, 0.25 mM of each dNTP, 1.25 U of Pfu Turbo polymerase, 1x Turbo reaction buffer and 0.2 µM forward and reverse primer containing the desired
mutation. Thermocycling was performed in a Mastercycler using the following conditions:

<table>
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<td>5-8 min</td>
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<td>68°C</td>
<td>5 min</td>
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</table>

After PCR the template plasmid was digested for 1 h at 37°C with 20 U Dpn1.

4.2. Purification of DNA

Following PCR and/or restriction digest, DNA was purified from Agarose gels using the NucleoSpin Gel and PCR Clean-up Kit (Machery-Nagel, Düren, Germany) according to the manufacturers recommendations. The final elution was performed with dH2O instead of the provided elution buffer.

5. MICROBIOLOGICAL METHODS

5.1. Transformation of E. coli

Chemically competent E. coli XL1 blue cells (50-100 µl) were thawed on ice. DNA was added to the cells (10-200 ng) and incubated on ice for 20 min. Cells were heat shocked for 60 s at 42°C, incubated for 2 min at 4°C and recovered for 1 h in 800 µl LB medium at 37°C. Then the cells were harvested by centrifugation for 3 min at 4,000 rpm, resuspended in 150 µl LB media and plated on selective LB plates containing either 100 µg/ml Ampicillin or 100 µg/ml Kanamycin. Cells were grown over night at 37°C.

Electro competent E. coli BL21(DE) cells containing the pRARE plasmid were thawed on ice and then transferred to a Gene Pulser Cuvette (0.2 cm gap, Bio-Rad). DNA was added to the cells (10-200 ng) and cells were transformed in the Gene Pulser Xcell system (Bio-Rad) at 2.5 kV. Cells
were kept on ice for 2 min, and recovered in LB for 1 h at 37°C and harvested for 3 min at 4,000 rpm. Then cells were either plated on LB plates containing 100 µg/ml Ampicillin/100 µg/ml Chloramphenicol or 100 µg/ml Kanamycin/100 µg/ml Chloramphenicol or grown in liquid LB media containing the respective antibiotics.

5.2. Transformation of yeast

Yeast cells were grown in liquid media at appropriate temperature to mid-log phase. 50 ml of cells were harvested by centrifugation at 2,300 rpm for 3 min. Cells were washed first in 50 ml dH2O and then with 5 ml LiSorb (100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 M Sorbitol). LiSorb was added to the pelleted cells to achieve a concentration of 1 OD600 per 10 µl. 50 µl of competent cells were added to an Eppendorf tube containing 10 µl denatured salmon sperm DNA (ssDNA; 10 mg/µl, Sigma-Aldrich) and 500 ng of transforming plasmid DNA. For genomic integration, 100 µl of competent cells, 10 µl of salmon sperm DNA and 1-2 µg of PCR product were used. For plasmid transformation 300 µl of LiPEG (100 mM lithium acetate, 10 mM Tris-HCl pH 8, 1 mM EDTA, 45% (w/v) PEG4000) was added to the cells, whereas for genomic integration 600 µl of LiPEG was applied onto the cells. Cells were incubated for 30 min at 25°C on a shaker at 1,400 rpm. 35 µl DMSO (70 µl for genomic integrations) was added to the cells. Cells were incubated for 15 min at 42°C, pelleted for 3 min at 2,000 rpm and washed in 2 ml dH2O. Then cells were recovered for 1 h at 30°C in non-selective media. After recovery, cells were washed in dH2O, plated on appropriate selection media, and grown at appropriate temperature for 2-4 days.

5.3. Isolation of genomic DNA from yeast

To check transformed stains for genomic integrations, cells were grown on appropriate plates at 30°C over night. A small amount of cells was transferred to a 1.5 ml screw-cap microcentrifuge tube containing glass beads (400-600 µm diameter) and 100 µl NTES buffer (10 mM Tris-HCl pH 8.0,
100 mM NaCl, 1 mM EDTA). Cells were lysed by vortexing (Disruptor Genie, Scientific Industries) at maximum speed for 8 min at 4°C. The lysate was cleared for 10 min at 14,000 rpm and 1 µl of the supernatant was used as a template for colony PCR reactions.

For genomic DNA extraction, yeast cells were grown in appropriate liquid media to saturation. 5 ml of the culture were harvested for 3 min at 5,000 rpm, resuspended in dH₂O and transferred to a 1.5 ml screw-cap microcentrifuge tube. Cells were pelleted at 14,000 rpm for 5 s and resuspended in 200 µl lysis solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM Na₂EDTA). Cells were lysed by vortexing (Disruptor Genie) at maximum speed for 3 min at 4°C in presence of glass beads (400-600 µm diameter). After addition of 200 µl Phenol-chloroform-isoamyl alcohol and 200 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA), the lysates were centrifuged for 2 min at 14,000 rpm. DNA in the supernatant was then precipitated by addition of 1 ml 100% ice-cold ethanol. The pellet was resuspended in 400 µl TE pH 8 and 3 µl RNaseA solution and incubated for 5 min at 37°C. 10 µl ammonium acetate in 1 ml 100% ethanol was added and genomic DNA was again pelleted at 14,000 rpm for 2 min. The pellet was air-dried and resuspended in 50 µl dH₂O. Phenol-chloroform-isoamyl alcohol extracted genomic DNA was used as PCR template for genes/inserts used for cloning.

5.4. Preparation of yeast whole cell extracts

Whole cell extracts were prepared by the alkaline lysis method. 10 ml cultures were grown to mid-log phase and then harvested by centrifugation for 3 min at 5,000 rpm. Cells were incubated on ice for 5 min and then lysed by the addition of 150 µl 1.85 M NaOH and 8% β-mercaptoethanol. After 10 min incubation on ice, 150 µl 50% TCA was added and samples were again incubated on ice for 10 min. The precipitates were pelleted at 14,000 rpm at 4°C for 10 min and washed with 1 ml ice-cold acetone. Pellets were solubilized in 100 µl 2x LDS sample buffer (Invitrogen), boiled for 10 min at 70°C and pelleted again prior to loading.
For preparing native yeast lysates for *in vitro* degradation assays, a WT strain was grown at 30°C to mid-log phase and 60 OD\textsubscript{600} units of cells were harvested. The cell pellet was resuspended in 1 ml lysis buffer (50 mM Tris-HCl pH 6.5, 50 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.15% NP40) and lysed with 0.4 g glass beads (400-600 µm diameter) using a vortex for 8 min at 4°C. The lysate was recovered and added to recombinant eS26.

6. **STRUCTURAL BIOLOGY**

6.1. **Protein expression and purification for NMR spectroscopy**

Uniformly \[^{15}\text{N}\]- and \[^{13}\text{C},^{15}\text{N}\]-labeled Tsr\textsubscript{21-152} was expressed in *E. coli* BL21 (DE3 +pRARE) cells as a C-terminal fusion to a (His)\textsubscript{6}-GB1 domain. The employed expression vector pEM1 was generated by subcloning the XbaI-BamHI fragment of the cell-free expression vector pCFX3 (Michel and Wüthrich, 2012) into pET19b (Novagen, Madison, WI, USA).

For the expression of Tsr\textsubscript{21-152} 10 ml from a 50 ml M9 pre-culture grown overnight with 100 µg/ml Ampicillin 100 µg/ml Chloramphenicol was used to inoculated 6 l pre-warmed M9 medium containing 3 g/l \[^{13}\text{C}\]-glucose (100 µg/ml Ampicillin 100 µg/ml Chloramphenicol). Cultures were grown to OD\textsubscript{600} of 0.5, then shifted from 37°C to 20°C. At OD\textsubscript{600} of ca. 0.8, cells were induced with 1 mM IPTG for 16 h. Cells were collected for 15 min at 5,000 rpm and resuspended in 30 ml buffer A (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 10 mM imidazole, 0.5 mM DTT) containing 1 mM DTT or 1 mM TCEP and passed five times at 75 psi through a M-110 L cell cracker (Microfluidics, Westwood, MA, USA). The supernatant was applied to a 5 ml HisTrap HP column (GE Healthcare) which was run at RT with a flow rate of 2 ml/min. 10 ml fractions were collected and the bound proteins eluted in a 100 ml gradient up to 100% buffer B (50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 500 mM imidazole, 0.5 mM DTT). Fractions were analyzed on SDS-PAGE and protein-containing fractions were pooled and dialyzed for 20 h at 4°C with 25 µM TEV in a 3.5 kDa SpectraPor3 (Spectrum Labs, Rancho Dominguez, CA, USA) against 4 l cleavage buffer (50 mM Tris-HCl, pH 7.4,
100 mM NaCl, 0.5 mM DTT). To remove the (His)$_6$-GB1 domain, TEV protease cleaved proteins were run over a 5 ml HisTrap HP column with a flow rate of 1 ml/min. Bound proteins were eluted with buffer B. 10 ml fractions were collected and analysed on SDS-PAGE. Protein containing fractions were pooled and concentrated. Concentrated sample was applied to a Superdex 75 HiLoad 16/60 (GE Healthcare) equilibrated in 20 mM NaPi-HCl at pH 7.0, 1 mM DTT, 10 µM EDTA. Gelfiltration was performed at a flow rate of 0.5 ml/min. Protein containing fractions were concentrated to an NMR sample with concentrations from 500 µM-1 mM. 5% D$_2$O was added.

6.2. NMR spectroscopy measurements and structure calculations

All NMR experiments were recorded at 293.15 K on Bruker Avance 500 MHz, 600 MHz, 700 MHz and 900 MHz spectrometers equipped with CryoProbe™ and triple resonance probes with shielded z-gradient coils (Bruker, Billerica, MA, USA). Quadrature-detection in the indirect dimensions was achieved by States time-proportional phase incrementation (Marion and Wüthrich, 1983). The water signal was suppressed with spin-lock pulses or WATERGATE (Piotto et al., 1992). The raw NMR data were processed with TOPSPIN 3.0 (Bruker). Proton chemical shifts are referenced to the water resonance and $^{13}$C and $^{15}$N chemical shifts are indirectly referenced to $^1$H using the absolute frequency ratios (Wishart et al., 1995). For spectral analysis the program CARA (www.nmr.ch) was used (Keller, 2004).

The backbone resonances of TSR2$^{1-152}$ were assigned using the 3D HNCA (Kay et al., 1990), 3D HNCACB (Kay et al., 1990) and 3D CBCA(CO)NH (Grzesiek and Bax, 1993) experiments. Side-chain resonances were assigned using the 3D (H)CC(CO)NH (Montelione et al., 1992), 3D H(CC)(CO)NH (Montelione et al., 1992) and 3D $[^{15}$N,1H]-HSQC-TOCSY (Marion et al., 1989) experiments. NOE-based distance constraints for the structure calculation were derived from 3D $^{15}$N-resolved $[^1$H, $^1$H]-NOESY, 3D aliphatic $^{13}$C-resolved $[^1$H, $^1$H]-NOESY and 3D aromatic $^{13}$C-resolved $[^1$H, $^1$H]-NOESY spectra (Ikura et al., 1990; Talluri and Wagner, 1996) which were all recorded with a mixing time of 60 milliseconds. The
3D NOESY spectra were further used to complete and confirm the assignment of all amide and side-chain resonances.

For the structure determination of TSR2\textsuperscript{1–152} we employed the ATNOS (Herrmann \textit{et al}., 2002b) procedure for automated peak picking and used the list of picked peaks from cycle two in combination with the chemical shift list from the sequence-specific resonance assignment and the 3D NOESY spectra as input for automated NOESY assignment and structure calculation in the program CYANA (Guntert, 2004; Herrmann \textit{et al}., 2002a). The final structure calculation in cycle 7 included only distance constraints that could be unambiguously assigned based on the calculated 3D structure from cycle 6. The 20 conformers with the lowest residual target function obtained from cycle 7 were then energy-minimized in implicit water using the program AMBER12 (Case \textit{et al}., 2012). We used the program PYMOL for the analysis of protein structures.

Heteronuclear \textsuperscript{15}N-\textsuperscript{1}H nuclear Overhauser effect (NOE) experiments of TSR2\textsuperscript{1–152} were recorded at 293.15 K and 700 MHz with a relaxation delay of 5 seconds.
**APPENDIX**

Table S1. New nomenclature for r-proteins. From Ban *et al.*, 2014.


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<td>HIS&lt;sup&gt;r&lt;/sup&gt;-GSP1AMP</td>
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<td>pEM1-SLX9</td>
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Table S3. Yeast strains used in this study.

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<td>this study</td>
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1 provided by E. Hurt, University of Heidelberg, Heidelberg, Germany
2 provided by J. Warner, Albert Einstein College of Medicine, Bronx, NY, USA
3 provided by M. Seedorf, University of Heidelberg, Heidelberg, Germany
4 provided by K. Karbstein, Scripps Research Institute, Jupiter, FL, USA

The secondary HRP-conjugated α-rabbit and α-mouse antibodies (Sigma-Aldrich, USA) were used at 1:1,000-1:5,000 dilutions.
Table S5. Peptides used for SRM assay.

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