

**Shining Light On Dim2:
Dissecting the Roles of an RNA-Binding Protein in
Ribosomal Small Subunit Assembly**

by

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DEDICATION

The work presented herein is dedicated to my parents who have always shown me nothing but the love and support that every person deserves. They taught me to chase my dreams, no matter how impossible circumstances may seem at times.

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The DMS probing experiments performed in Chapter IV were carried out by Katrin Karbstein; all other experiments were done by Heather Woolls.

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List of Keywords and Abbreviations

SSU: Small Subunit

LSU: Large Subunit

rRNA: ribosomal RNA

UTP: U Three Associated Protein Complexes

ITS1: Internal Transcribed Spacer 1

Nob1: Endonuclease involved in D-site cleavage

Dim2: Partner of Nob1

KH: K-Homology

D3/K: Triple Aspartic Acid Mutation in Dim2

HR/E: Histidine, Arginine Double Mutation in Dim2

GxxG: Replacement of the NSWT Loop for GxxG Residues in Dim2

CHAPTER I

Introduction

Structure and Function of Mature Ribosomes

Ribosomes are responsible for all protein synthesis. Composed of a large subunit (LSU) and a small subunit (SSU), referred to as 60S and 40S respectively, eukaryotic ribosomes contain four ribosomal RNAs (rRNAs; about 5500 nucleotides) and 78 ribosomal proteins (r-proteins; Figure 1). To carry out protein synthesis, ribosomes must translate the sequence of nucleotides contained in messenger RNAs (mRNAs) into polypeptides. The small ribosomal subunit serves as the decoding site in protein synthesis where the three-nucleotide codons of the mRNAs are paired with complementary anticodons of amino-acylated transfer RNAs (tRNAs). The large ribosomal subunit provides the catalyst that drives peptide bond formation between amino acids on adjacent tRNAs. While until recently only medium-resolution ($\sim 9\text{\AA}$) cryo-EM structures of eukaryotic ribosomes were available (Chandramouli *et al.*, 2008), high-resolution x-ray structures of bacterial ribosomes have been used to fit the cryo-EM maps of eukaryotic ribosomes to give pseudo atomic resolution (Spahn *et al.*, 2001). This is possible because of the substantial homology between bacterial and eukaryotic rRNA and r-proteins. Most recently this technique has been pushed to reveal a 5.5\AA structure of eukaryotic ribosomes (Armache *et al.*, 2010), and a crystal structure at 4.15\AA has been published (Ben-Shem *et al.*, 2010). Despite the conservation of both the structure and the function of ribosomes there are significant differences between bacterial and eukaryotic ribosomes assembly.

Bacterial ribosomes can be reconstituted *in vitro* under specific conditions (Bubunenko *et al.*, 2006; Culver & Noller, 2000; Culver, 2003), and only a few assembly factors have been identified that are essential for assembly *in vivo*. In contrast, eukaryotic ribosomes have not been reconstituted *in vitro*, and ribosome biogenesis *in vivo* requires approximately 200 essential assembly factors (Bernstein *et al.*, 2004a; Connolly & Culver, 2009; Fromont-Racine *et al.*, 2003; Maki & Culver, 2005; Saveanu *et al.*, 2003; Strunk & Karbstein, 2009), including an assortment of enzymes as well as a plethora of structural proteins. Mature ribosomes produce proteins at an impressive rate to meet cellular

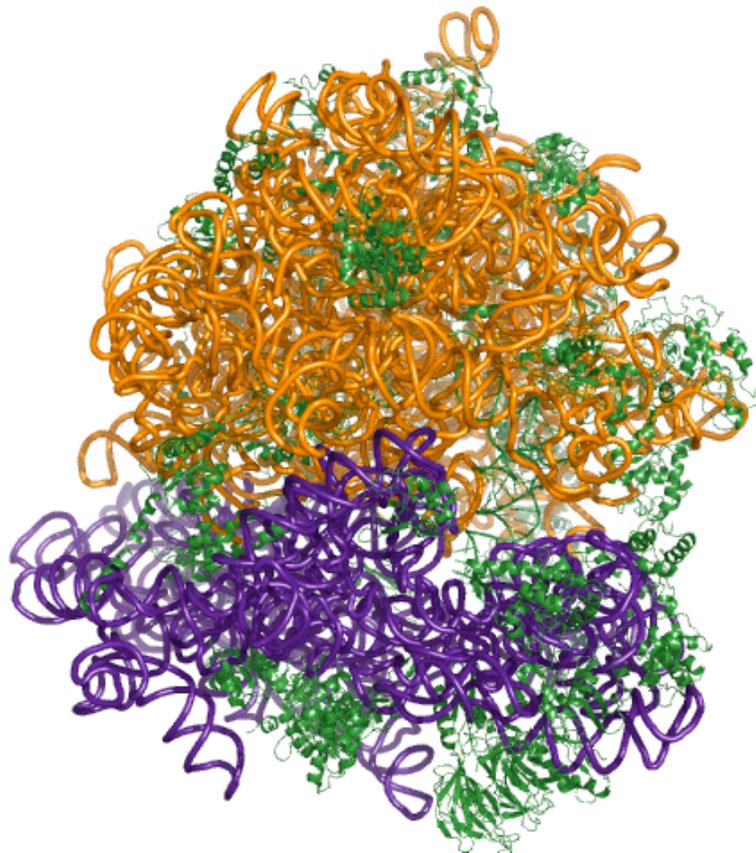


Figure 1: Structure of *S. cerevisiae* ribosome. The crystal structure of the ribosome reveals a small subunit (18S rRNA in purple with r-proteins in green) and a large subunit (25S rRNA in orange with r-proteins in green). PDB files used are 3JYV, 3JYW and 3JYX.

demands and with astonishing fidelity. Both of these features require the integrity of the whole particle as they require long-range communication through the particle. This suggests that assembly has to be highly efficient and correct. Small mishaps in assembled ribosomes could drastically affect the ability of ribosomes to meet the demands of living cells.

Assembly of Functional Ribosomes

Assembly of eukaryotic ribosomes involves the transcription of the four rRNAs and incorporation of the 78 r-proteins into mature ribosomes. While the 5S rRNA is transcribed separately by RNA polymerase III, three of the four rRNAs (18S, 5.8S and 25S rRNAs) are cotranscribed in the nucleolus as a single transcript by RNA polymerase I. During transcription assembly factors and ribosomal proteins associate with the precursor rRNA (pre-rRNA), which can be visualized as “terminal balls” in chromatin spreads. These structures consist of associating assembly factors and are collectively known as the SSU processosomes, which include the U Three Assoiated Proteins (UTP)-A, UTP-B and UTP-C complexes, the U3 snoRNA and additional U3 associated proteins, including the Imp3, Imp4, Mpp10 complex, as well as other proteins, including Dim1 and Dim2 (Bernstein *et al.*, 2004a; Champion *et al.*, 2008; Dragon *et al.*, 2002; Freed *et al.*, 2010; Gallagher & Baserga, 2004; Gallagher *et al.*, 2004; Lee & Baserga, 1999). These proteins are required for co-transcriptional cleavages at sites A_0 and A_1 and at A_2 , which separates the LSU and SSU RNAs (Figure 2) and releases the terminal balls. Assembly factors included in the SSU

processosome, as in other pre-ribosomal particles, include methylases, pseudouridylases, and nucleases, but also helicases, as well as GTPases and ATPases.

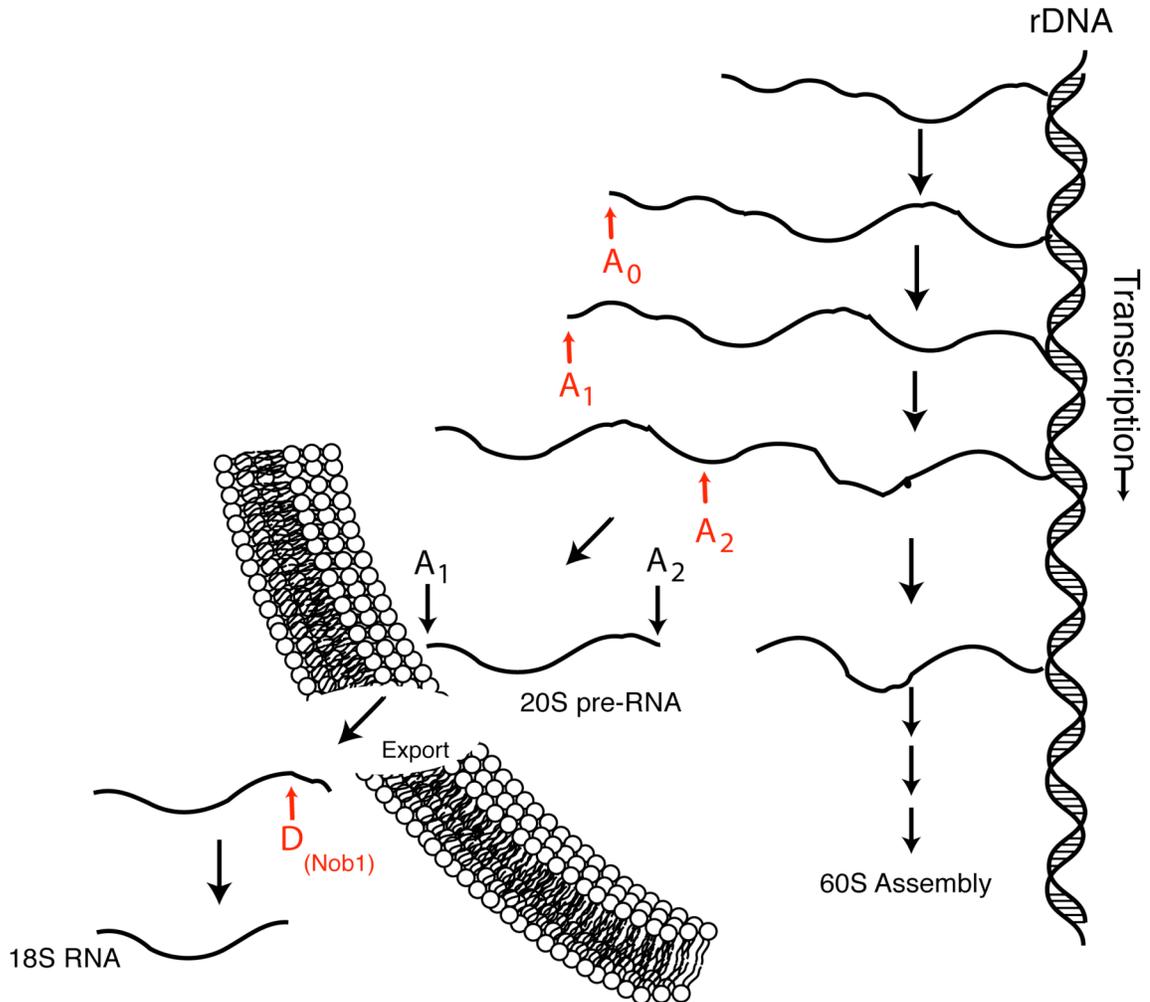


Figure 2: rRNA processing involves many steps and additional assembly factors. Transcription and early processing steps take place in the nucleolus. Following nuclear export, late cytoplasmic maturation of the small subunit includes D-site cleavage.

Following export of the nascent subunits cytoplasmic maturation of the small subunit precursor includes methylation of two conserved adenosines by the methylase Dim1, D-site cleavage (shown in Figure 2) by the endonuclease Nob1, as well as incorporation of the final r-proteins.

Towards Understanding the Role of Assembly Factors

Assembly Factors are Essential for Ribosome Biogenesis in Eukaryotes

Because bacterial ribosomes can be reconstituted from the purified components, it was not expected that assembly required many additional factors. Early hints that this expectation might be false came from the observation that bacterial assembly intermediates increase in size as they near completion, reflecting the increasing number of bound r-proteins. In contrast, in eukaryotes early precursors are substantially larger than the mature particle because they contain assembly factors, which dissociate as assembly progresses, leading to smaller sizes of later assembly intermediates. Consistent with this finding, a few temperature-sensitive yeast strains were isolated that were deficient in ribosome assembly. However, it was not until 1986 that the first factor required for ribosome assembly, Rrp1, was cloned followed by about 15-17 assembly factors over the next ~ 10 years (Horsey *et al.*, 2004).

The complexity of the ribosome assembly machinery did not become clear until the first ribosome assembly factors were TAP-tagged and the co-purifying proteins identified via mass-spectrometry. This strategy led, in rapid succession, to the definition of several assembly intermediates along the pathways leading to formation of 40S and 60S ribosomes (Gavin *et al.*, 2002; Gong *et al.*, 2009; Grandi *et al.*, 2002). In addition, this research also demonstrated that assembly of both subunits was completely separate, as virtually no proteins were identified that associated with and required for both assembling subunits.

Along the 40S assembly pathway this analysis suggested an early assembly intermediate, the SSU processosome containing the Utp proteins, plus other proteins such as Dim1, Dim2, Rrp5, shown in Figure 3a (Bernstein *et al.*, 2004b; Eppens *et al.*, 1999; Lafontaine *et al.*, 1998; Vanrobays *et al.*, 2004b; Vos *et al.*, 2004). In addition to this early assembly intermediate, this analysis suggested a later intermediate, which contained the 20S rRNA, the direct precursor to mature 18S rRNA, as well as the assembly factors, Enp1, Ltv1, Tsr1, Nob1, Dim1 and Dim2, shown in Figure 3b (Chen *et al.*, 2003; Gelperin *et al.*, 2001; Lamanna & Karbstein, 2009; Seiser *et al.*, 2006). Surprisingly, both Dim1 and Dim2, already found in early assembling ribosomes, are the only proteins known to be involved in both early as well as late 40S assembly steps.

Assembly Factors Associate with Pre-ribosomes as Subcomplexes

While the identification of new ribosome assembly factors is now virtually complete, with only few additional factors being discovered, the next frontier was to define subcomplexes within these large assembling ribosomes to define functional units.

In a comprehensive approach to this question, high-speed ultracentrifugation of affinity-purified complexes followed by mass spectrometry was used to determine subcomplexes that remain intact even after prolonged centrifugation. This approach defined the UTP-A, UTP-B, and UTP-C subcomplexes of the SSU processosome (Figure 3a). UTP-A had been previously defined because of the requirement of these constituent proteins for rRNA synthesis. Similarly, the UTP-C subcomplex has since been linked biochemically to transcription of r-proteins.

Aside from small subunit assembly complexes, the Erb1/Ytm1/Nop7

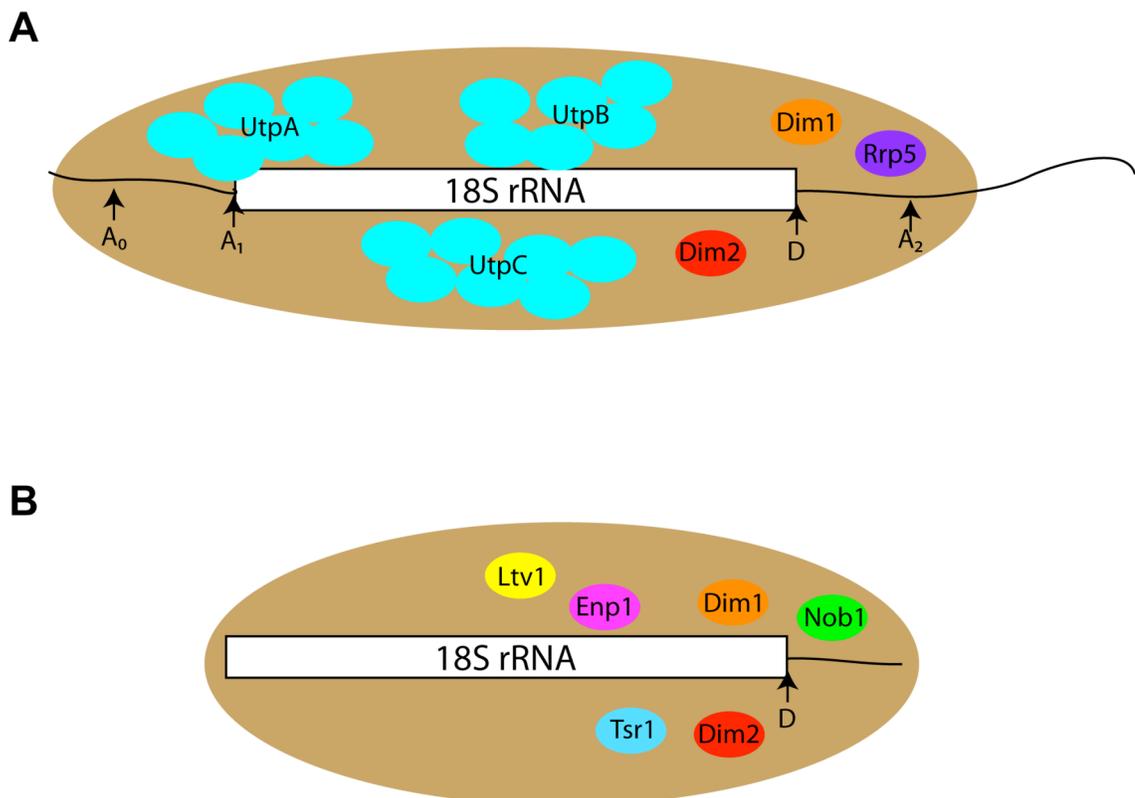


Figure 3: Assembly factors associate with small subunit precursor. A) early processing intermediates of the small subunit involve Utp complexes as well as proteins such as Dim1, Dim2, and Rrp5. B) the late processing intermediate of the small subunit assembly pathway involves late association assembly factors Ltv1, Enp1, Tsr1, Dim1, Dim2, and Nob1.

subcomplex was identified via this methodology to be involved in large subunit assembly (Miles *et al.*, 2005; Tang *et al.*, 2008), and later confirmed in detailed biochemical experiments to play roles in 25S rRNA processing as well as incorporation of two large subunit r-proteins.

Similarly, another well-defined subcomplex involved in ribosome biogenesis is the 5S rRNA complex, which is required for incorporation of 5S rRNA into maturing ribosomes (Zhang *et al.*, 2007). Mature 5S rRNA (transcribed separately) binds to the ribosomal protein rpL5. Along with ribosomal protein rpL11, this complex is then recruited to pre-ribosomes via assembly factors Rpf2 and Rrs1.

Mapping the Topology of Predefined Complexes Involved in Ribosome Assembly

While these data show that essential assembly factors associate with rRNA in subcomplexes (as well as individually), defining the role of individual assembly factors is becoming the focus of much ribosome assembly research. While some assembly factors appear to have enzymatic activity, the majority of the many assembly factors that contain only nucleic acid binding domains and/or protein-protein interaction motifs. Because these domains have no experimentally testable activities, determining the function of many of these essential assembly factors has remained elusive. To begin to address this problem a number of labs have started to map the interactions within the subcomplexes described above with the idea that visualizing the location of assembly factors could lead to a better understanding of their function.

GST-pull down experiments and two-hybrid assays were used to dissect protein-protein interactions in the Erb1/Ytm1/Nop7 subcomplex involved in large subunit processing. Interactions between protein-interactions domains of each protein subcomplex was mapped, as shown in Figure 4 (Miles *et al.*, 2005; Tang *et al.*, 2008). The

C-terminal WD40 domain of Ytm1 interacts with the N-terminal conserved region of Erb1. The interaction

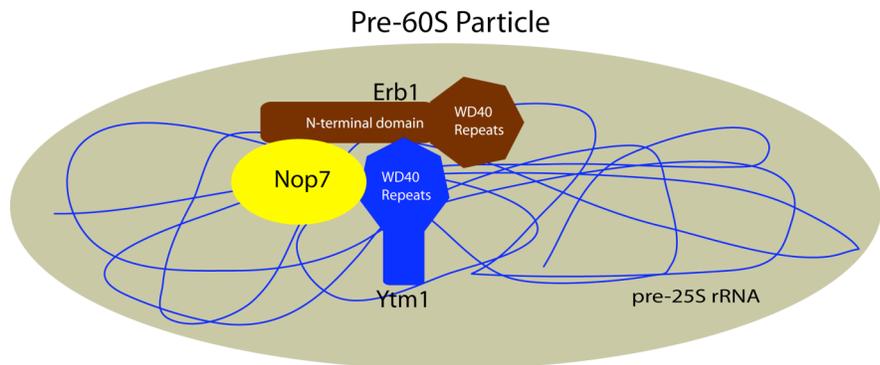


Figure 4: The Erb1/Ytm1/Nop7 complex is involved in large subunit maturation. The WD40 domains of Ytm1 interact with Nop7 and the N-terminal domain of Erb1. Nop7 also interacts with the N-terminal domain of Erb1. All three proteins are involved in the binding of the complex to the large subunit precursor.

between Erb1 and Nop7 was mapped to the central region of Nop7. Furthermore, research showed an interdependence of the members of the Erb1/Ytm1/Nop7 subcomplex in incorporation of the complex into pre-ribosomes; when one protein is depleted or the interactions between these specific domain interactions are disrupted, the recruitment and localization of the other proteins in the complex are affected.

On the 40S pathway, using a systematic yeast 2-hybrid analysis, research began mapping all the protein-protein interactions within the UTP-A and UTP-B subcomplexes involved in early processing of the small subunit, shown in Figure 5 (Freed & Baserga, 2010; Freed *et al.*, 2010). The resulting interaction map for the UTP-A complex is shown in Figure 5a. This work suggests that Utp8, previously suggested to be also an RNA-binding protein, is at the heart of the UTP-A complex, with direct interactions to almost every other of the UTP-A subcomplex (Champion *et al.*, 2008; Champion *et al.*, 2009). Interestingly, Utp8, and Utp9 are also implicated in tRNA export out of the nucleus, indicating a link between tRNA and rRNA maturation.

Using the same yeast two-hybrid system, the protein-protein interactions of the UTP-B complex (Utp1, Utp6, Utp12, Utp13, Utp12, and Utp21) were determined,

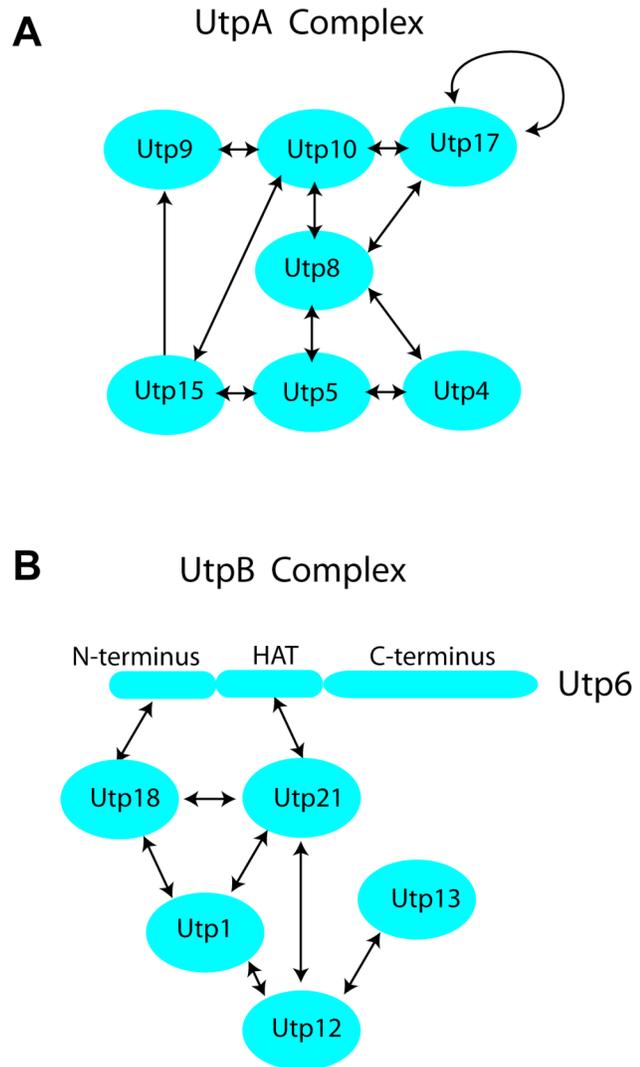


Figure 5: Protein-protein interactions of the UtpA and UtpB complexes were determined by yeast two-hybrid assays. A) Protein-protein interactions are shown between the Utp proteins of the UtpA complex. B) protein-protein interactions are shown between the UtpB proteins.

shown in Figure 5b. Again, one protein, Utp21, is shown to form direct interactions with all but one other protein. Additional work on the UTP-B complex demonstrates that the half- α -tetratricopeptide repeat, or HAT, domain of Utp6 interacts with a specific ligand peptide from Utp21 (Champion *et al.*, 2009). This protein-protein interaction between assembly factors is essential for efficient early processing of rRNA and was the first example of ligand determination for a HAT domain.

Towards the same goal of mapping the binding sites of assembly factors, as well as provide topological constraints for the structure of these pre-ribosomal particles, the Tollervey lab developed a novel UV-crosslinking technique termed CRAC (UV crosslinking and cDNA analysis; Granneman *et al.*, 2010). Using this technique, which uses UV light to crosslink proteins to RNA followed by reverse transcription and subsequent sequencing of the product, they were able to determine where proteins bound to rRNA. Since the binding of U3 snoRNP complex had been greatly dissected previously, they were able to use U3 snoRNP as a proof of principle. U3 snoRNP assists in the proper folding of rRNA and has been shown to form transient basepairs with pre-rRNA, and has an obligatory role in the early processing steps A₀ and A₁ cleavages in the 5' external transcribed spacer (5' ETS) as well as A₂ cleavage in the internal transcribed spacer 1 (ITS1). Using UV light, the snRNP proteins Nop1, Nop56, Nop58, and Rrp9 were crosslinked to U3 snoRNA as well as rRNA, and the bound sequences identified by reverse transcription and sequencing (Granneman *et al.*, 2009). Using CRAC, the interactions between the assembly

factors and both RNAs (U3 and rRNA) were detected suggesting that these assembly factors could be involved in recruiting the snoRNA to pre-ribosomes (Granneman *et al.*, 2009).

Also using the CRAC technique, the binding sites of six out of seven assembly factors stably bound to the last precursor to 40S particles were revealed (Granneman *et al.*, 2010). Crosslinks consistent with a single binding site were seen for Dim1, Enp1, and Rio2. For Dim1 these were also consistent with previous crosslinks obtained with the bacterial homolog, KsgA. Furthermore, Enp1 was previously shown to form a salt-labile complex with Ltv1 and Rps3, an r-protein, which binds on the solvent side of the beak structure on pre-ribosomes (Chen *et al.*, 2003). Consistent with this finding, Enp1 maps to the solvent side of the beak. In contrast, binding sites for Tsr1, Ltv1 and Nob1 appear to be more spread out and are hard to reconcile with a single pre-ribosomal intermediate. This can be explained by the nature of the technique which crosslinks whole-cell lysates, and could thus produce crosslinks in a large number of different species found throughout the assembly pathway. Furthermore, the crosslink for Nob1 does not reflect the previously obtained footprinting data, which show Nob1 protects the 3'-end of 18S rRNA (Lamanna & Karbstein, 2009). The seventh late-associated assembly factor Dim2 did not produce any crosslinks, likely because the C-terminal tag was cleaved prior to incorporation into pre-ribosomal particles (B. S. Strunk & K. Karbstein, unpublished observations).

In a complementary approach, Campbell and Karbstein mapped the direct protein-protein interactions between late cytoplasmic assembly factors as well as

a subset of small subunit ribosomal proteins (Campbell & Karbstein, 2011). Using recombinantly purified proteins, direct protein-protein interactions were determined by retention of untagged proteins on an amylose resin through interactions with MBP-tagged proteins, shown in Figure 6a. Their data show that the late cytoplasmic assembly factors are associated as two basic modules. One module is composed of Enp1 and Ltv1, and based on prior data, binds near the beak. The second module is located on the subunit interface side of the head region (Figure 6b) and is defined by direct interactions between the endonuclease Nob1, the kinase Rio2 and Dim2. The GTPase-like Tsr1, and the methylase Dim1 are also associated with this module. As a result of this research, models were proposed that approximate the location of these assembly factors on pre-ribosomes based on interactions with assembly factors and ribosomal proteins of known location. Additionally, the kinase Rio2 is implicated as a possible “master regulator” of these late processing steps, as it interacts directly or indirectly with every other assembly factor.

Taken together these data show that for some assembly intermediates mapping of subcomplexes has provided valuable insight into the organization of pre-ribosomal particles. However, these data have not yet been used to take the next step and dissect the functional role of interactions between individual assembly factors.

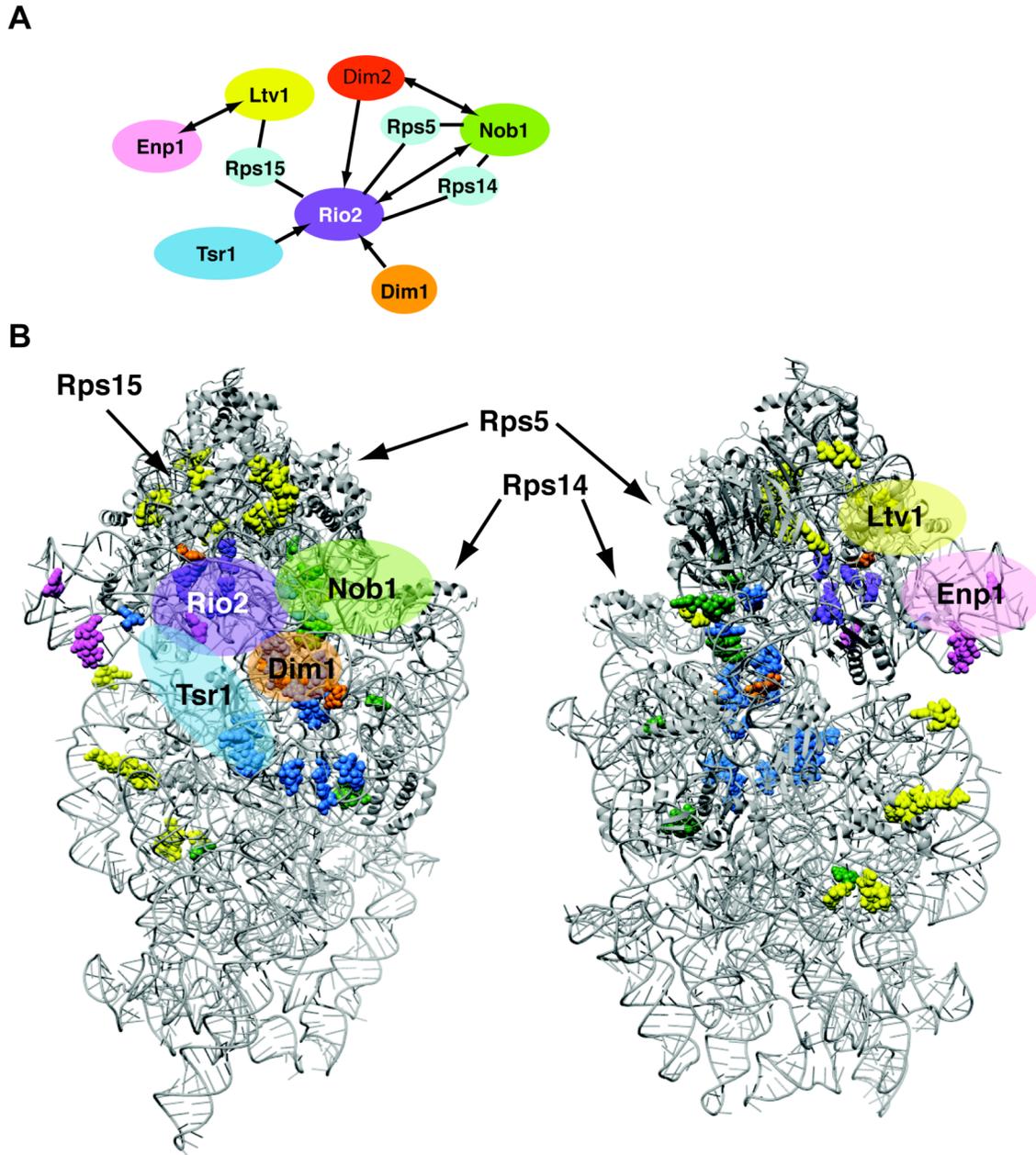


Figure 6: A model of late associated assembly factors shows two modules. A) a protein-protein interaction map shows interactions between the late assembly factors and several ribosomal proteins. B) Based on the protein-protein interactions, protein binding sites were modeled onto pre-ribosomes to show two modules involved in late cytoplasmic maturation.

Dissecting the Functional Role of Individual Assembly Factors

Much work on ribosome assembly factors has shown that the majority of assembly factors are essential, and directly involved in ribosome biogenesis of individual subunits. However, since depletion of individual assembly factors typically interferes with several steps in ribosome synthesis, likely because the ribosome assembly intermediates are highly cooperatively built as suggested by the large number of protein-protein interactions within the studied modules (Figures 4, 5 and 6), determining the specific role of individual assembly factors has remained fairly elusive.

The GTPase Bms1 Aids Assembly of Processing Complexes

The Bms1/Rcl1 subcomplex is the only complex for which the function of an interaction has been mapped so far (Dutca & Culver, 2005; Gelperin *et al.*, 2001). Bms1 is an essential GTPase that initially had been described as a component of the U3 snoRNA complex with a suggested interaction with the putative nuclease Rcl1, shown through a yeast two-hybrid screen. Shortly thereafter, Bms1 was shown to bind directly to Rcl1 as well as U3 snoRNA (Gelperin *et al.*, 2001). Using the interdependence of Bms1 binding to Rcl1 and U3 snoRNA in the presence of GTP versus GDP, a model was proposed for the role of Bms1 in ribosome assembly. When GTP is bound Bms1 has a higher affinity for Rcl1, suggesting a nucleotide-dependence regulatory interaction with the putative nuclease. Binding of Rcl1 to GTP-Bms1 results in an increase in affinity for U3 snoRNA. An interaction with the U3 snoRNA complex allows for

delivery of the Rcl1-GTP-Bms1 subcomplex to pre-ribosomes. Following hydrolysis of GTP on Bms1 through an internal domain conformational change, a decrease in affinity between Rcl1 and Bms1-GDP results in dissociation of Bms1-GDP from pre-ribosomes. This model shows how Bms1 uses the binding of GTP to form an assembly complex and the hydrolysis of GTP to deliver Rcl1 to pre-ribosomes. This model also shows how the GTP activity of Bms1 could serve as a regulatory step in the activity of a putative nuclease.

Nob1 is the Endonuclease Involved in Late Cytoplasmic D-Site Cleavage

Nob1 contains a PIN domain, which has been found in several nucleases, and binds directly to *in vitro* transcribed analogs of 3'-end pre-18S rRNA (Fatica *et al.*, 2004; Lamanna & Karbstein, 2009; Lamanna & Karbstein, 2011). Depletion of Nob1 leads to an accumulation of 20S rRNA *in vivo* and Nob1 purified from yeast or *E. coli* cleaves RNA substrate analogs, strongly implicating Nob1 as the endonuclease responsible for D-site cleavage. Interestingly, Nob1 is associated with early, nucleolar pre-40S particles that serve as the substrate for D-site cleavage reaction, suggesting that regulation of Nob1 activity prevents premature, nucleolar D-site cleavage. One aspect of this regulation has been recently dissected and is shown to be due to an RNA conformational change (Lamanna & Karbstein, 2011). Prior to A₂ cleavage the region of helix 44 containing the decoding site is not fully formed but instead replaced by an alternative duplex formed between a strand of helix 44 and a segment of the Internal Transcribed Spacer 1 (ITS1; shown in purple in Figure 7). Nob1 binds to

the rRNA in this structure, albeit in a different conformation, in which it is unable to cleave the pre-rRNA. Cleavage at site A_2 removes the region of ITS1 that is duplexed with part of the helix 44 leading to a conformational change in which helix 44 is completed. Nob1 is repositioned on this rRNA structure, and now is able to cleave pre-rRNA, shown in Figure 7.

While this switch is an essential regulator of Nob1 it is likely not the only regulated step, as data suggest that the switch is closely associated with export,

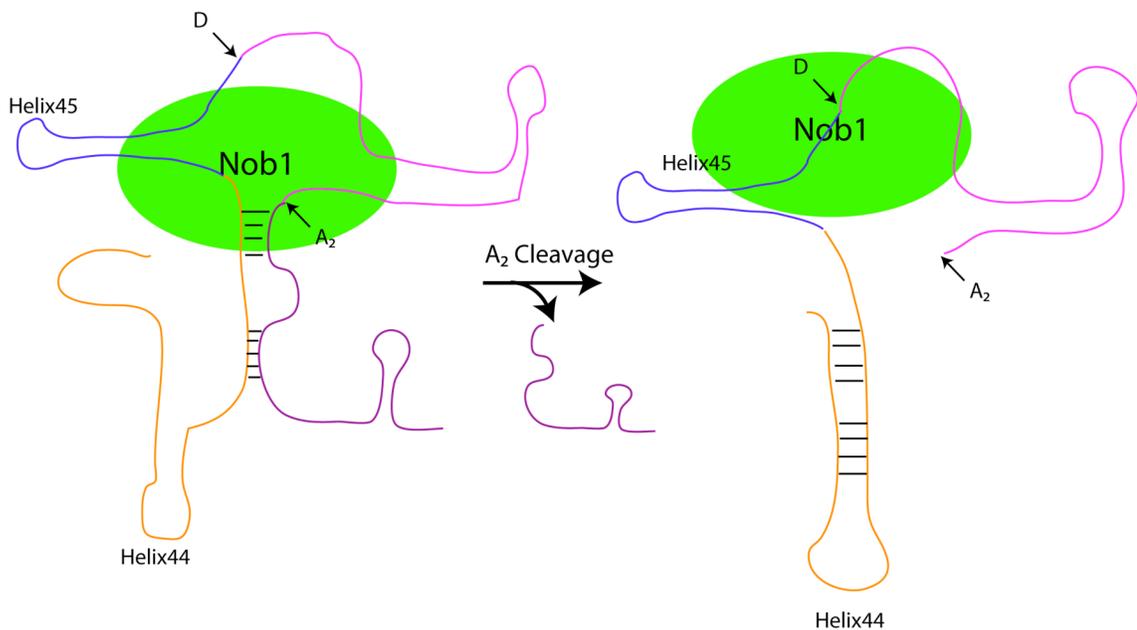


Figure 7: Nob1 binds differently to pre- and post- A_2 cleavage rRNA conformations. Research has shown that helix 44 is not completely formed prior to A_2 cleavage; instead an alternative helix is formed between part of helix 44 and ITS1. Following A_2 cleavage, ITS1 is removed and helix 44 is completely formed. Nob1 binds differently to these two structures with binding to post- A_2 conformation centered around D-cleavage site.

and the Nob1-bound pre-rRNA accumulates in the cytosol. Thus we wanted to know if other proteins are involved in regulating Nob1 activity. Nob1 binds directly to only two assembly factors, the kinase Rio2 and the RNA binding protein Dim2 (Campbell & Karbstein, 2011). This latter interaction is also supported by earlier yeast two-hybrid assays. Interestingly, an interaction between Dim2 and Rio2

was also shown, suggesting Dim2 as a possible regulator of either Rio2 kinase activity and/or Nob1 cleavage activity.

*Dim2, an RNA Binding Protein, is an Assembly Factor Involved in 40S Assembly
and Interacts with Nob1*

Initial research showed Dim2 is a component of the small subunit processome and is associated early in the nucleolus (Vanrobays *et al.*, 2004a). However, whereas most components of nucleolar processing dissociate from the small subunit precursor prior to export from the nucleus, Dim2 remains bound and is additionally associated with late cytoplasmic maturation (Vanrobays *et al.*, 2004a; Vanrobays *et al.*, 2008a). Consistent with this, research with GFP-labeled Dim2 showed that Dim2 shuttles between the nucleus and the cytoplasm, a process that is nutrition-dependent. Dim2 has a nuclear export sequence in the C-terminal region suggesting Dim2 could play a role in export of the small subunit precursor (Vanrobays *et al.*, 2008b).

Sequence alignments of Dim2 suggest the presence of a C-terminal KH domain, a common RNA binding domain. When regions of this domain or the entire domain were deleted binding to rRNA was completely abrogated, as shown through gel-shift electrophoretic-mobility assays. Additionally, when this domain was deleted *in vivo* loss of early rRNA processing resulted in the formation of the 35S and 33S rRNAs, and a subsequent loss of 20S and 18S rRNAs, similar to a Dim2 deletion, showing that this C-terminal KH domain is required for Dim2 binding to rRNA, a step that is essential for Dim2 function.

Recently, the structure of an archeal Dim2, aDim2p, complexed with the initiation factor a/eIF2 and a fragment of the 3' end of the 16S rRNA was solved (Jia *et al.*, 2007). In this structure the C-terminal KH domain appears bound to a sequence of rRNA that is universally conserved, suggesting a conserved role for this KH domain. Additionally, the second KH domain of aDim2p appears bound to the Shine-Dalgarno sequence of the 16S rRNA on one side and bound to a/eIF2 on the other side, suggesting that Dim2 could play a role in translation initiation. However, considering that eukaryotic organisms do not have Shine-Dalgarno sequences, these data suggest that the function of this KH domain could be somehow altered throughout evolution.

With suggested roles in 1) early rRNA processing steps, 2) late cytoplasmic steps, 3) export of the small subunit precursor from the nucleus, 4) recruitment of Dim1 to pre-ribosomes, 5) a possible role in initiation, and 6) the suggested interaction with Nob1, the specific function of Dim2 remains vague.

In this thesis, I have focused on further dissecting the role of Dim2 in ribosome assembly and specifically in understanding the role of the interaction between Dim2 and the Nob1 nuclease for ribosome assembly. This is the first example of dissecting the ribosome assembly role of a protein containing only protein-RNA interaction domains and sets the stage for developing methodology to dissect how assembly factors only containing nucleic acid binding domains can regulate dynamic enzymatic assembly factors.

References

- Ben-Shem, A., Jenner, L., Yusupova, G., & Yusupov, M. (2010). Crystal structure of the eukaryotic ribosome. *Science (New York, N.Y.)*, 330(6008), 1203-1209. doi:10.1126/science.1194294
- Bernstein, K. A., Gallagher, J. E., Mitchell, B. M., Granneman, S., & Baserga, S. J. (2004a). The small-subunit processome is a ribosome assembly intermediate. *Eukaryotic Cell*, 3(6), 1619-1626. doi:10.1128/EC.3.6.1619-1626.2004
- Bernstein, K. A., Gallagher, J. E., Mitchell, B. M., Granneman, S., & Baserga, S. J. (2004b). The small-subunit processome is a ribosome assembly intermediate. *Eukaryotic Cell*, 3(6), 1619-1626. doi:10.1128/EC.3.6.1619-1626.2004
- Bubunenko, M., Korepanov, A., Court, D. L., Jagannathan, I., Dickinson, D., Chaudhuri, B. R., . . . Culver, G. M. (2006). 30S ribosomal subunits can be assembled in vivo without primary binding ribosomal protein S15. *RNA (New York, N.Y.)*, 12(7), 1229-1239. doi:10.1261/rna.2262106
- Champion, E. A., Kundrat, L., Regan, L., & Baserga, S. J. (2009). A structural model for the HAT domain of Utp6 incorporating bioinformatics and genetics. *Protein Engineering, Design & Selection : PEDS*, 22(7), 431-439. doi:10.1093/protein/gzp022
- Champion, E. A., Lane, B. H., Jackrel, M. E., Regan, L., & Baserga, S. J. (2008). A direct interaction between the Utp6 half- α -tetratricopeptide repeat domain and a specific peptide in Utp21 is essential for efficient pre-rRNA processing.

Molecular and Cellular Biology, 28(21), 6547-6556. doi:10.1128/MCB.00906-08

Chandramouli, P., Topf, M., Menetret, J. F., Eswar, N., Cannone, J. J., Gutell, R. R., . . . Akey, C. W. (2008). Structure of the mammalian 80S ribosome at 8.7 Å resolution. *Structure (London, England : 1993)*, 16(4), 535-548. doi:10.1016/j.str.2008.01.007

Chen, W., Bucaria, J., Band, D. A., Sutton, A., & Sternglanz, R. (2003). Enp1, a yeast protein associated with U3 and U14 snoRNAs, is required for pre-rRNA processing and 40S subunit synthesis. *Nucleic Acids Research*, 31(2), 690-699.

Connolly, K., & Culver, G. (2009). Deconstructing ribosome construction. *Trends in Biochemical Sciences*, 34(5), 256-263. doi:10.1016/j.tibs.2009.01.011

Culver, G. M. (2003). Assembly of the 30S ribosomal subunit. *Biopolymers*, 68(2), 234-249. doi:10.1002/bip.10221

Culver, G. M., & Noller, H. F. (2000). In vitro reconstitution of 30S ribosomal subunits using complete set of recombinant proteins. *Methods in Enzymology*, 318, 446-460.

Dragon, F., Gallagher, J. E., Compagnone-Post, P. A., Mitchell, B. M., Porwancher, K. A., Wehner, K. A., . . . Baserga, S. J. (2002). A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature*, 417(6892), 967-970. doi:10.1038/nature00769

- Dutca, L. M., & Culver, G. M. (2005). Regulation of rRNA processing: A role for a unique GTPase. *Molecular Cell*, *20*(4), 497-499.
doi:10.1016/j.molcel.2005.11.006
- Eppens, N. A., Rensen, S., Granneman, S., Raue, H. A., & Venema, J. (1999). The roles of Rrp5p in the synthesis of yeast 18S and 5.8S rRNA can be functionally and physically separated. *RNA (New York, N.Y.)*, *5*(6), 779-793.
- Fatica, A., Tollervey, D., & Dlakic, M. (2004). PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. *RNA (New York, N.Y.)*, *10*(11), 1698-1701.
doi:10.1261/rna.7123504
- Freed, E. F., & Baserga, S. J. (2010). The C-terminus of Utp4, mutated in childhood cirrhosis, is essential for ribosome biogenesis. *Nucleic Acids Research*, *38*(14), 4798-4806. doi:10.1093/nar/gkq185
- Freed, E. F., Bleichert, F., Dutca, L. M., & Baserga, S. J. (2010). When ribosomes go bad: Diseases of ribosome biogenesis. *Molecular bioSystems*, *6*(3), 481-493. doi:10.1039/b919670f
- Fromont-Racine, M., Senger, B., Saveanu, C., & Fasiolo, F. (2003). Ribosome assembly in eukaryotes. *Gene*, *313*, 17-42.
- Gallagher, J. E., & Baserga, S. J. (2004). Two-hybrid Mpp10p interaction-defective Imp4 proteins are not interaction defective in vivo but do confer specific pre-rRNA processing defects in *saccharomyces cerevisiae*. *Nucleic Acids Research*, *32*(4), 1404-1413. doi:10.1093/nar/gkh318
- Gallagher, J. E., Dunbar, D. A., Granneman, S., Mitchell, B. M., Osheim, Y., Beyer, A. L., & Baserga, S. J. (2004). RNA polymerase I transcription and

- pre-rRNA processing are linked by specific SSU processome components. *Genes & Development*, 18(20), 2506-2517. doi:10.1101/gad.1226604
- Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., . . . Superti-Furga, G. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*, 415(6868), 141-147. doi:10.1038/415141a
- Gelperin, D., Horton, L., Beckman, J., Hensold, J., & Lemmon, S. K. (2001). Bms1p, a novel GTP-binding protein, and the related Tsr1p are required for distinct steps of 40S ribosome biogenesis in yeast. *RNA (New York, N.Y.)*, 7(9), 1268-1283.
- Gong, Y., Kakihara, Y., Krogan, N., Greenblatt, J., Emili, A., Zhang, Z., & Houry, W. A. (2009). An atlas of chaperone-protein interactions in *Saccharomyces cerevisiae*: Implications to protein folding pathways in the cell. *Molecular Systems Biology*, 5, 275. doi:10.1038/msb.2009.26
- Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., . . . Hurt, E. (2002). 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. *Molecular Cell*, 10(1), 105-115.
- Granneman, S., Kudla, G., Petfalski, E., & Tollervey, D. (2009). Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. *Proceedings of the National Academy of Sciences of the United States of America*, 106(24), 9613-9618. doi:10.1073/pnas.0901997106

- Granneman, S., Petfalski, E., Swiatkowska, A., & Tollervey, D. (2010). Cracking pre-40S ribosomal subunit structure by systematic analyses of RNA-protein cross-linking. *The EMBO Journal*, *29*(12), 2026-2036.
doi:10.1038/emboj.2010.86
- Horsey, E. W., Jakovljevic, J., Miles, T. D., Harnpicharnchai, P., & Woolford, J. L., Jr. (2004). Role of the yeast Rrp1 protein in the dynamics of pre-ribosome maturation. *RNA (New York, N.Y.)*, *10*(5), 813-827.
- Jia, M. Z., Ohtsuka, J., Lee, W. C., Nagata, K., & Tanokura, M. (2007). Crystal structure of Dim2p: A preribosomal RNA processing factor, from *pyrococcus horikoshii* OT3 at 2.30 Å. *Proteins*, *69*(2), 428-432. doi:10.1002/prot.21381
- Lafontaine, D. L., Preiss, T., & Tollervey, D. (1998). Yeast 18S rRNA dimethylase Dim1p: A quality control mechanism in ribosome synthesis? *Molecular and Cellular Biology*, *18*(4), 2360-2370.
- Lamanna, A. C., & Karbstein, K. (2009). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(34), 14259-14264. doi:10.1073/pnas.0905403106
- Lamanna, A. C., & Karbstein, K. (2011). An RNA conformational switch regulates pre-18S rRNA cleavage. *Journal of Molecular Biology*,
doi:10.1016/j.jmb.2010.09.064
- Lee, S. J., & Baserga, S. J. (1999). Imp3p and Imp4p, two specific components of the U3 small nucleolar ribonucleoprotein that are essential for pre-18S rRNA processing. *Molecular and Cellular Biology*, *19*(8), 5441-5452.

- Maki, J. A., & Culver, G. M. (2005). Recent developments in factor-facilitated ribosome assembly. *Methods (San Diego, Calif.)*, 36(3), 313-320.
doi:10.1016/j.ymeth.2005.04.008
- Miles, T. D., Jakovljevic, J., Horsey, E. W., Harnpicharnchai, P., Tang, L., & Woolford, J. L., Jr. (2005). Ytm1, Nop7, and Erb1 form a complex necessary for maturation of yeast 66S preribosomes. *Molecular and Cellular Biology*, 25(23), 10419-10432. doi:10.1128/MCB.25.23.10419-10432.2005
- Saveanu, C., Namane, A., Gleizes, P. E., Lebreton, A., Rousselle, J. C., Noaillac-Depeyre, J., . . . Fromont-Racine, M. (2003). Sequential protein association with nascent 60S ribosomal particles. *Molecular and Cellular Biology*, 23(13), 4449-4460.
- Seiser, R. M., Sundberg, A. E., Wollam, B. J., Zobel-Thropp, P., Baldwin, K., Spector, M. D., & Lycan, D. E. (2006). Ltv1 is required for efficient nuclear export of the ribosomal small subunit in *saccharomyces cerevisiae*. *Genetics*, 174(2), 679-691. doi:10.1534/genetics.106.062117
- Spahn, C. M., Beckmann, R., Eswar, N., Penczek, P. A., Sali, A., Blobel, G., & Frank, J. (2001). Structure of the 80S ribosome from *saccharomyces cerevisiae*--tRNA-ribosome and subunit-subunit interactions. *Cell*, 107(3), 373-386.
- Strunk, B. S., & Karbstein, K. (2009). Powering through ribosome assembly. *RNA (New York, N.Y.)*, 15(12), 2083-2104. doi:10.1261/rna.1792109
- Tang, L., Sahasranaman, A., Jakovljevic, J., Schleifman, E., & Woolford, J. L., Jr. (2008). Interactions among Ytm1, Erb1, and Nop7 required for assembly of

the Nop7-subcomplex in yeast preribosomes. *Molecular Biology of the Cell*, 19(7), 2844-2856. doi:10.1091/mbc.E07-12-1281

Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., & Lafontaine, D. L.

(2004a). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. *RNA (New York, N.Y.)*, 10(4), 645-656.

Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., & Lafontaine, D. L.

(2004b). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. *RNA (New York, N.Y.)*, 10(4), 645-656.

Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., &

Lafontaine, D. L. (2008a). TOR regulates the subcellular distribution of DIM2, a KH domain protein required for cotranscriptional ribosome assembly and pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.

doi:10.1261/rna.1176708

Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., &

Lafontaine, D. L. (2008b). TOR regulates the subcellular distribution of DIM2, a KH domain protein required for cotranscriptional ribosome assembly and pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.

doi:10.1261/rna.1176708

Vos, H. R., Bax, R., Faber, A. W., Vos, J. C., & Raue, H. A. (2004). U3 snoRNP and Rrp5p associate independently with *saccharomyces cerevisiae* 35S pre-rRNA, but Rrp5p is essential for association of Rok1p. *Nucleic Acids Research*, 32(19), 5827-5833. doi:10.1093/nar/gkh904

- Yusupov, M. M., Yusupova, G. Z., Baucom, A., Lieberman, K., Earnest, T. N., Cate, J. H., & Noller, H. F. (2001). Crystal structure of the ribosome at 5.5 Å resolution. *Science (New York, N.Y.)*, 292(5518), 883-896.
doi:10.1126/science.1060089
- Zhang, J., Harnpicharnchai, P., Jakovljevic, J., Tang, L., Guo, Y., Oeffinger, M., Woolford, J. L., Jr. (2007). Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. *Genes & Development*, 21(20), 2580-2592. doi:10.1101/gad.1569307

CHAPTER II

In vitro Characterization of Dim2

Introduction

Mature, functional eukaryotic ribosomes that can carry out protein synthesis consist of 4 ribosomal RNAs (rRNAs) and approximately 78 proteins (r-proteins). To produce functional ribosomes, these rRNAs and proteins must be synthesized and assembled. In addition to the rRNAs and r-proteins, roughly 200 additional proteins as well as about 70 RNAs are required to properly assemble mature ribosomes (Fatica & Tollervey, 2002; Ferreira-Cerca *et al.*, 2005; Ferreira-Cerca *et al.*, 2007; Fromont-Racine *et al.*, 2003). Included in these additional assembly proteins are enzymes that catalyze modifications, rearrangements and cleavages of the rRNAs (Osheim *et al.*, 2004). Not surprisingly, among the many ribosome assembly factors are RNA binding proteins.

In addition to facilitating and potentially regulating ribosome assembly, RNA-binding proteins are used for a range of cellular functions from the transcription of DNA into mRNAs, rRNAs, and tRNAs to modification and processing of these initial transcripts, export into the cytoplasm for translation, translation regulation, and stability. While RNA binding proteins have a myriad of functions and must be able to specifically bind their substrates, there are few RNA binding domains (RBDs). These include RNA Recognition Motifs (RRMs),

KH domains, Zn finger motifs, and OB-fold domains (Clery *et al.*, 2008; Flynn & Zou, 2010; Grishin, 2001; Klug, 2010; Maris *et al.*, 2005; Valverde *et al.*, 2008).

KH-domains (hnRNP K- Homology) were first discovered in heterogeneous nuclear ribonucleoprotein K and have since been revealed in a wide-array of proteins involved in RNA metabolism and function (Chmiel *et al.*, 2006; Du *et al.*, 2008; Siomi *et al.*, 1993; Wen *et al.*, 2010). Based on sequence alignments and structural analyses, a minimal $\beta\alpha\alpha\beta$ motif comprising approximately 70 amino acids was defined. Additionally, a canonical GxxG loop is conserved between the two α -helices, in which the xx residues are typically

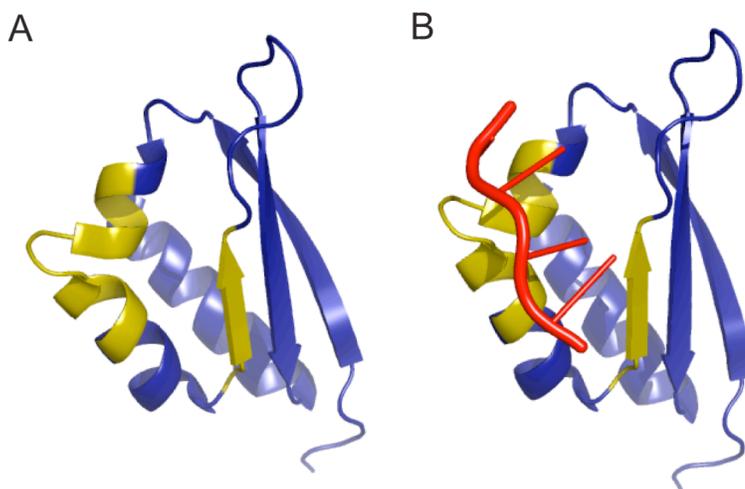


Figure 8. Nucleic acid binding site on KH domains. A) The KH domain of hnRNP K is shown with the RNA binding interface highlighted in yellow. B) The KH domain of hnRNP K is shown bound to single-stranded DNA. The structures shown were derived from PDB entrant 1zzj.

charged. The structures of many KH-domains have been solved either in solution or by x-ray crystallography. Furthermore, some structures of KH-domains bound to RNA have been

solved and NMR experiments have provided further information about an RNA binding surface common to these domains (Backe *et al.*, 2005; Braddock *et al.*, 2002; Du *et al.*, 2005; Du *et al.*, 2007; Gopal *et al.*, 2001; Valverde *et al.*, 2007). These data suggest that the $\beta 2$ - $\alpha 1$ - $\alpha 2$, GxxG and variable loop make up a

conserved RNA binding surface (Figure 8A). The RNA-binding groove is large enough for approximately 4 bases, and structures of KH domains bound to substrates show 3-4 bases bound to each KH domain (Figure 8B). Further specificity is gained by additional secondary structure elements adjacent to the KH domain or simply adding subsequent KH domains. Correspondingly, binding experiments with the KH domain-containing proteins Nova 2 and KSRP defined 6 nucleotides sequences that were specifically bound through a C-terminal extension in addition to the KH domain (Garcia-Mayoral *et al.*, 2008; Jensen *et al.*, 2000; Musunuru & Darnell, 2004).

Dim2 is an essential yeast ribosome assembly factor conserved from archaea to humans, which contains a canonical C-terminal KH domain that has been shown to bind rRNA (Vanrobays *et al.*, 2004; Vanrobays *et al.*, 2008b). In this chapter, we analyze the structural domains of Dim2 and its interaction with the D-site endonuclease Nob1, using a combination of sequence analysis tools as well as biochemical experiments. Our data show that Dim2 contains three KH domains. We show that Dim2 dimerizes via an N-terminal KH domain, interacts with Nob1 via the middle KH domain, and binds rRNA through the C-terminal KH domain.

Results

The domain structure of Dim2

Previous research showed that Dim2 bound rRNA through a canonical KH domain located in the C-terminal third of the protein sequence (Vanrobays *et al.*, 2008b; Fig. 9a). We wanted to verify the function of this KH domain and

investigate the role of the remaining two-thirds of the protein sequence in the role of Dim2 in ribosome assembly. The domain finder software SMART (Schultz *et al.*, 1998, Letunic *et al.*, 2009) suggests that Dim2 contains only one defined domain, a C-terminal KH domain. However, using the Jpred or PredictProtein secondary structure predictions software (Cole *et al.*, 2008, Rost *et al.*, 2004), we noticed the possibility of two additional KH domains contained within the first two-thirds of the sequence.

Residues 87-178 contain the $\beta 1$ - $\alpha 1$ - $\alpha 2$ - $\beta 2$ minimal motif present in KH domains (Figure 9B); however, the loop sequence between the $\alpha 1$ and $\alpha 2$ helices is NSWT instead of a classic GxxG loop. Sequence alignments of this domain (residues 87-178 of *S. cerevisiae* Dim2) with other known KH domains, including the C-terminal KH domain Dim2 (Figure 9B), show the conservation of key residues. Since residues 87-178 contain a noncanonical loop between the $\alpha 1$

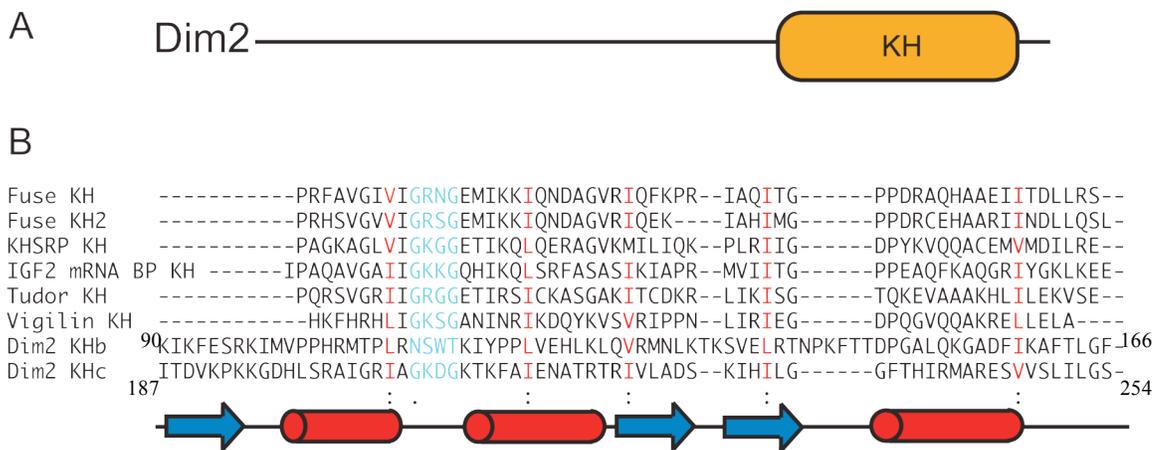


Figure 9. Sequence alignment of Dim2 predicts three KH domains. A) Previous research determined a C-terminal KH domain in Dim2. B) A sequence alignment of Dim2 across kingdoms shows high sequence conservation.

and $\alpha 2$ helices, we refer to this domain as a KH-like domain.

To support the notion that Dim2's central domain is a KH-like domain, we modeled the sequence of Dim2 using SwissModel and 3Djigsaw (Bates 2001, Arnold *et al.*, 2009; Figure 10a). Both programs BLAST the submitted sequence against all structures submitted to the PDB and model the sequence to the most homologous structure. Both programs modeled the Dim2 sequence to the

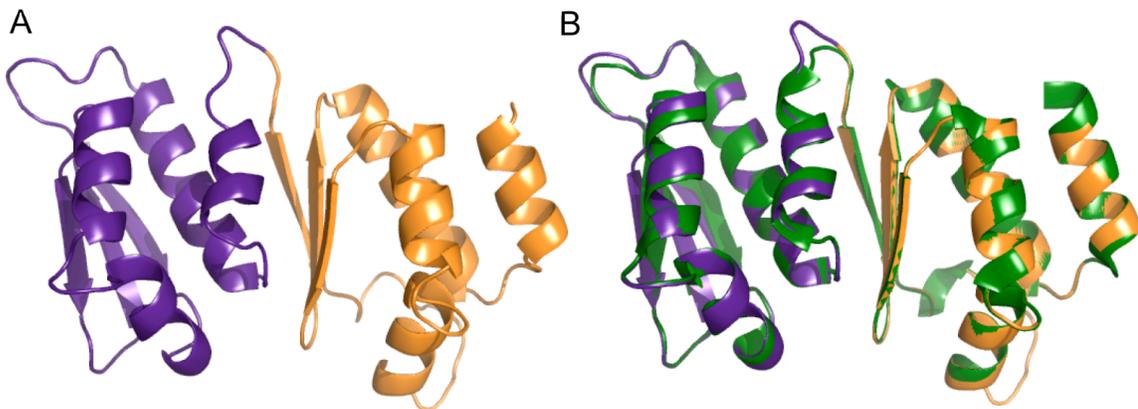


Figure 10. Model of Dim2. A) *S. cerevisiae* Dim2 modeled to the crystal structure of an archeal Dim2 (aDim2p). B) the superimposition of the modeled Dim2 (in purple and orange) with the crystal structure of aDim2p (green) shows high similarities.

archeal Dim2 structure, PDB entry 2E3U (*Pyrococcus horikoshii* OT3), aligning the predicted KH-like domain of Dim2 with the N-terminal KH domain of the archeal protein. Figure 10b shows the model of Dim2 (in colors purple and orange) aligned with the structure of the archeal protein (in green), showing minor differences between the two proteins. As expected the NSWT sequence aligns with the GxxG loop of the archeal protein. Furthermore, the Dim2 model shows three beta sheets that align well with the archeal structure, as well as the helix-GxxG loop-helix structure typical of KH domains. Additionally, the sequence homology between the yeast and archeal Dim2 proteins is ~80% (data not

shown). Taken together, these data support the presence of a noncanonical KH-like domain in Dim2.

Additionally, the first third of the protein sequence (residues 1-86) contains regions of low complexity with weak secondary structure predictions. However, there does appear to be a $\beta 1$ - $\alpha 1$ - $\alpha 2$ - $\beta 2$ minimal motif, including a classic GxxG loop between the two the α -helices, suggesting a putative KH domain at the N-terminus of Dim2. To test if the ability to form a putative KH domain at the N-terminus was conserved in other organisms we aligned the *S. cerevisiae* with homologs from protozoans, flies, vertebrates, and humans using Clustal W. Figure 11 shows high conservation in the KH-like and KH domains across a wide array of organisms. While the conservation in the N-terminal domain is much reduced, critical elements of KH-domains are conserved, including the GxxG loop, which is preserved in most organisms. This analysis suggests that Dim2 contains three domains: a strongly predicted C-terminal KH-domain, a central KH-like domain, which differs from canonical KH domains by the substitution of NSWT for the GxxG loop, as well as an N-terminal KH-domain, with weak secondary structure predictions.

The N-terminal domain is required for Dim2 dimerization

To dissect the function of these three domains, we cloned the full-length protein, as well as truncation constructs, which delete the N-terminal or C-terminal domains, into the pSV272 expression vector (Figure 12). Expressed in *E. coli* as MBP-fusion proteins, the constructs were purified as described in the Materials and Methods. Size-exclusion chromatography revealed that full-length

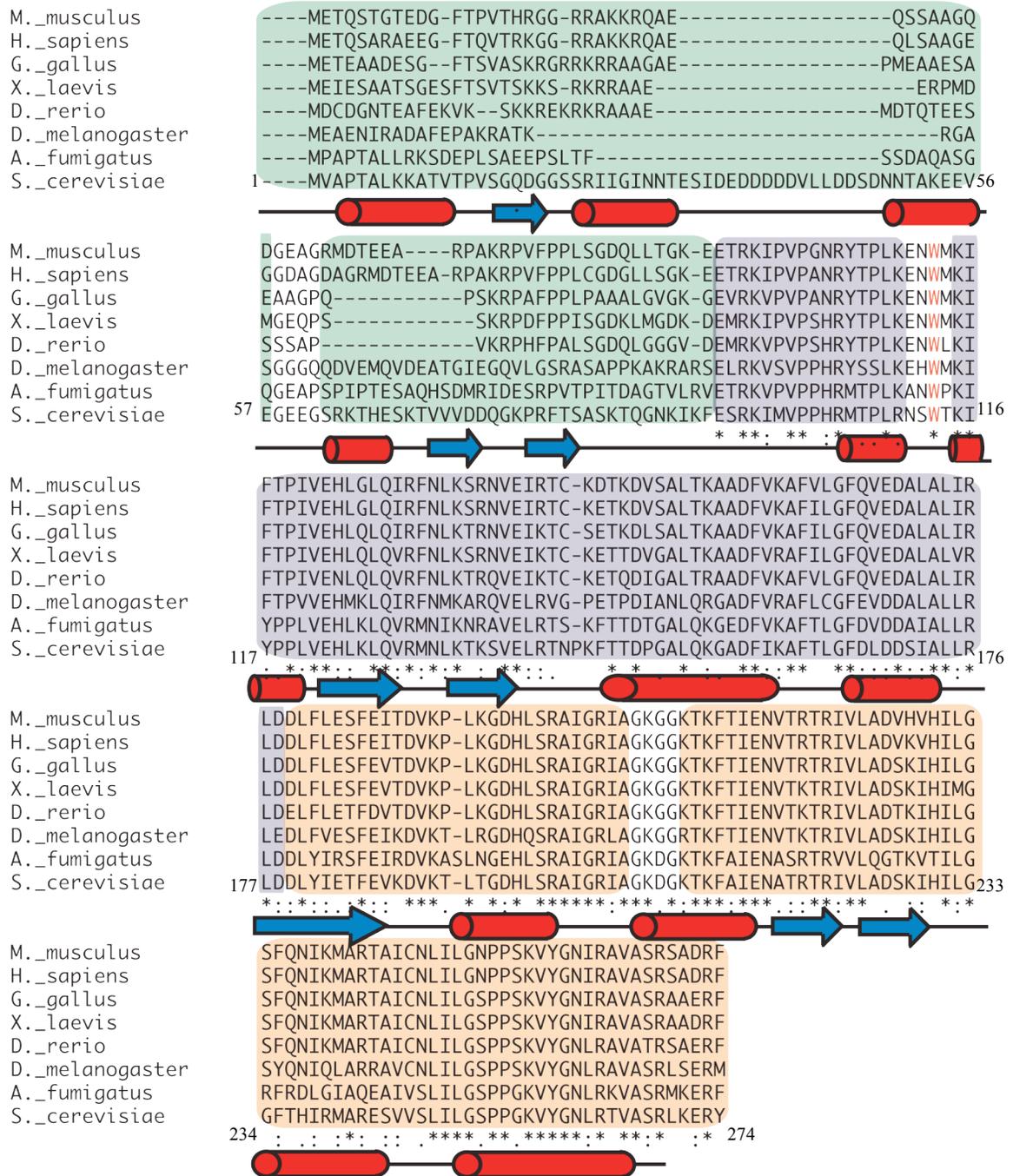


Figure 11. Alignment of Dim2 sequence across many domains of life show high conservation. A weakly conserved N-terminal KH domain is shown in green. A highly conserved KH domain lacking a canonical GxxG loop is shown in purple. A conserved tryptophan used in mutagenesis studies is highlighted in red. The highly conserved C-terminal KH domain involved in binding rRNA is shown in orange.

Dim2 elutes at a volume corresponding to ~65kD based on comparison to standards of known size (Figure 13). Given the expected molecular weight of 31

kDa, this size is indicative of a dimer of the full-length protein. Deletion of the C-terminal KH domain (Dim2 Δ C, shown in green in Figure 13) results in a shift in the peak, so that the Dim2 Δ C protein elutes at a volume corresponding to a ~45kD protein. Given the calculated molecular weight of 20 kDa, this is also consistent with the formation of a Dim2 dimer. In contrast, deleting the N-terminal KH domain

(Dim2 Δ N, shown in orange) shifts the peak much further despite the almost identical molecular weight (expected MW= 20.5 kDa), corresponding to a 20 kDa protein, strongly indicating that Dim2 Δ N is a monomer. While the elution volume during size exclusion chromatography is both a function of the molecular weight and the shape, the strong correlation between the dimeric molecular weight for the wild type and Dim2 Δ C proteins, as well as the perfect correlation with the monomeric molecular weight for the Dim2 Δ N, indicates strongly that Dim2 is a dimer, and that dimerization is mediated by the putative N-terminal KH domain.

The central KH-like domain binds the endonuclease Nob1

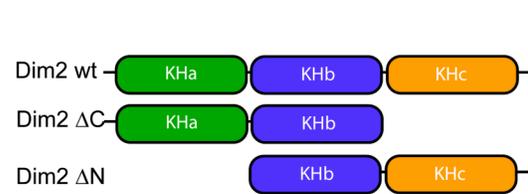
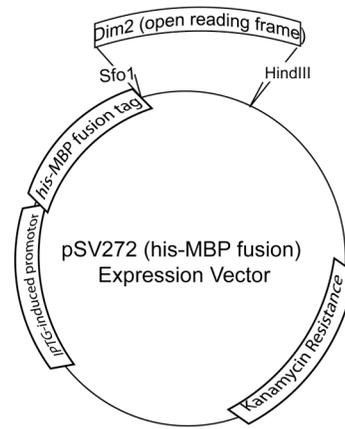


Figure 12. Cloned Dim2 constructs. A) Dim2 constructs were cloned into the MBP-fusion vector pSV272. B) constructs of Dim2 were cloned by truncating the N-terminal and C-terminal KH domains.

Prior research suggested that Dim2 and Nob1 might interact with each other, as an interaction was found in a yeast 2-hybrid assay (Tone & Toh-E, 2002). Furthermore, Nob1 was also co-precipitated with Dim2 in yeast. We wanted to test if this interaction is a direct protein-protein interaction between Dim2 and Nob1 and not mediated by concomitant association with the same pre-ribosomal particles. MBP-tagged Nob1 was overexpressed and purified from *E. coli* as previously described (Lamanna & Karbstein, 2009). Using MBP-tagged Nob1 bound to amylose resin, we tested for an interaction with the Dim2 constructs via *in vitro* pull down assays, as described in the Materials and Methods section. Figure 14a shows that full length Dim2 is retained when incubated with MBP-Nob1 bound to an amylose resin but not to the amylose resin or MBP alone. This finding shows that the interaction between Dim2 and Nob1 is direct and not mediated by other ribosome assembly factors or the pre-ribosomal complex.

To delineate which of the domains in Dim2 is responsible for the interaction with Nob1, we performed truncation analysis. Figure 14b shows that both the N-terminal and C-terminal truncations were also retained when incubated with MBP-Nob1 but not with the

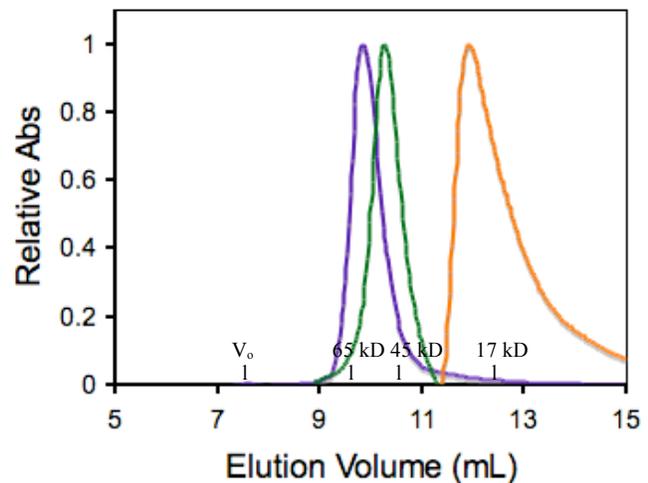


Figure 13. N-terminal KH domain is required for dimerization. Full length Dim2 (purple), as well as the C-terminal KH domain truncation (green), eluted as a dimer with size-exclusion chromatography. Whereas the N-terminal truncation (orange) resulted in an elution volume corresponding to a monomer size.

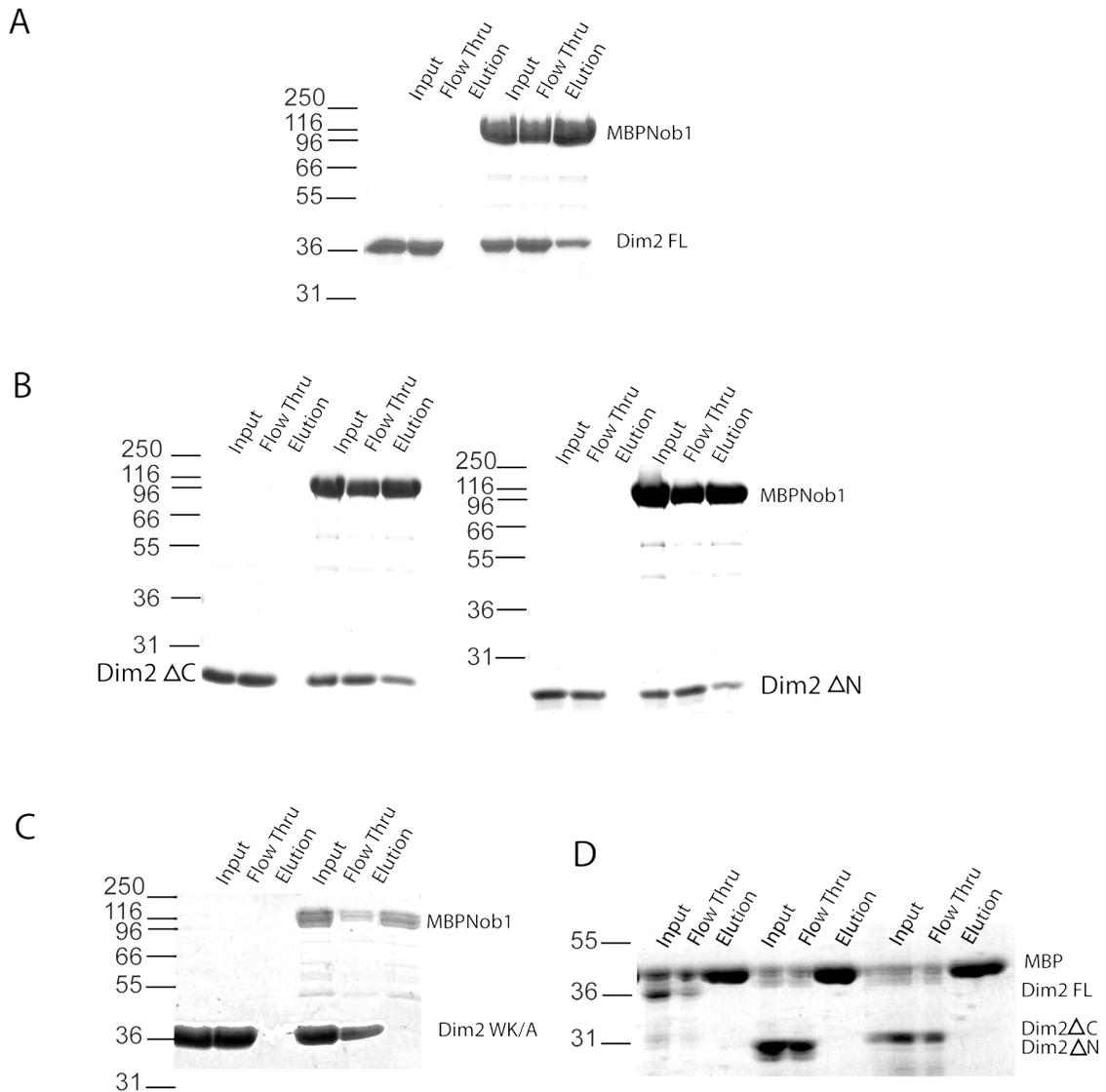


Figure 14. Dim2 interacts with Nob1 via the middle KH domain. A) pull down assays show an interaction between full length Dim2 and MBP-tagged Nob1 but no interaction between Dim2 and the amylose resin. B) pull down assays show an interaction between the N-terminal KH truncation and Nob1 as well as an interaction between C-terminal KH truncation and Nob1. C) The interaction between Dim2 and Nob1 is disrupted in the double mutant W113A/K115A. D) None of the Dim2 constructs bind to MBP alone.

amylose resin alone. These data indicate that the Dim2 central KH-like domain interacts with Nob1.

To further confirm that the central KH-like domain provides the interface for binding Nob1, we mutated residues in the central KH-like domain and then tested the recombinant protein for binding to Nob1. To design mutations, we

inspected sequence alignments and the predicted structure for Dim2. We noticed that the NSWT, which substitutes for the GxxG loop in the central KH-like domain, was conserved with the tryptophan universally conserved (highlighted in red in Figure 11). Based on the model, the side chain of W113 could pack against lysine 113; we therefore mutated this region, by converting tryptophan 113 and lysine 115 to alanines (referred to as WK/A). Figure 14c shows that mutating these two residues disrupts the Nob1-Dim2 interaction in the *in vitro* pull-down system. Together, these data indicate that the interaction between Nob1 and Dim2 is direct and mediated through the central KH-like domain of Dim2.

The C-terminal KH domain binds rRNA

Using truncation analysis, as well as point mutations, it has been shown that the C-terminal KH domain of Dim2 is required for binding to rRNA (Vanrobays, *et al.*, 2008a). We wanted to verify this finding and test the ability of our Dim2 truncations to bind rRNA. Using gel mobility shift assays, we tested the ability of the full-length, N- and C-terminal truncations to bind radiolabelled rRNA transcribed as described in Materials and Methods. As expected, full length Dim2 protein bound rRNA (Figure 15). Furthermore, as also expected, Dim2 Δ C does not bind the rRNA even at concentrations of 10 μ M. Consistent with previous data, these findings show that the C-terminal KH domain is required for Dim2 binding to rRNA. In contrast to the C-terminal deletion, Dim2 Δ N binds rRNA (Figure 15) with almost unchanged affinity. This result shows that neither the N-terminal domain nor dimerization are required to bind rRNA.

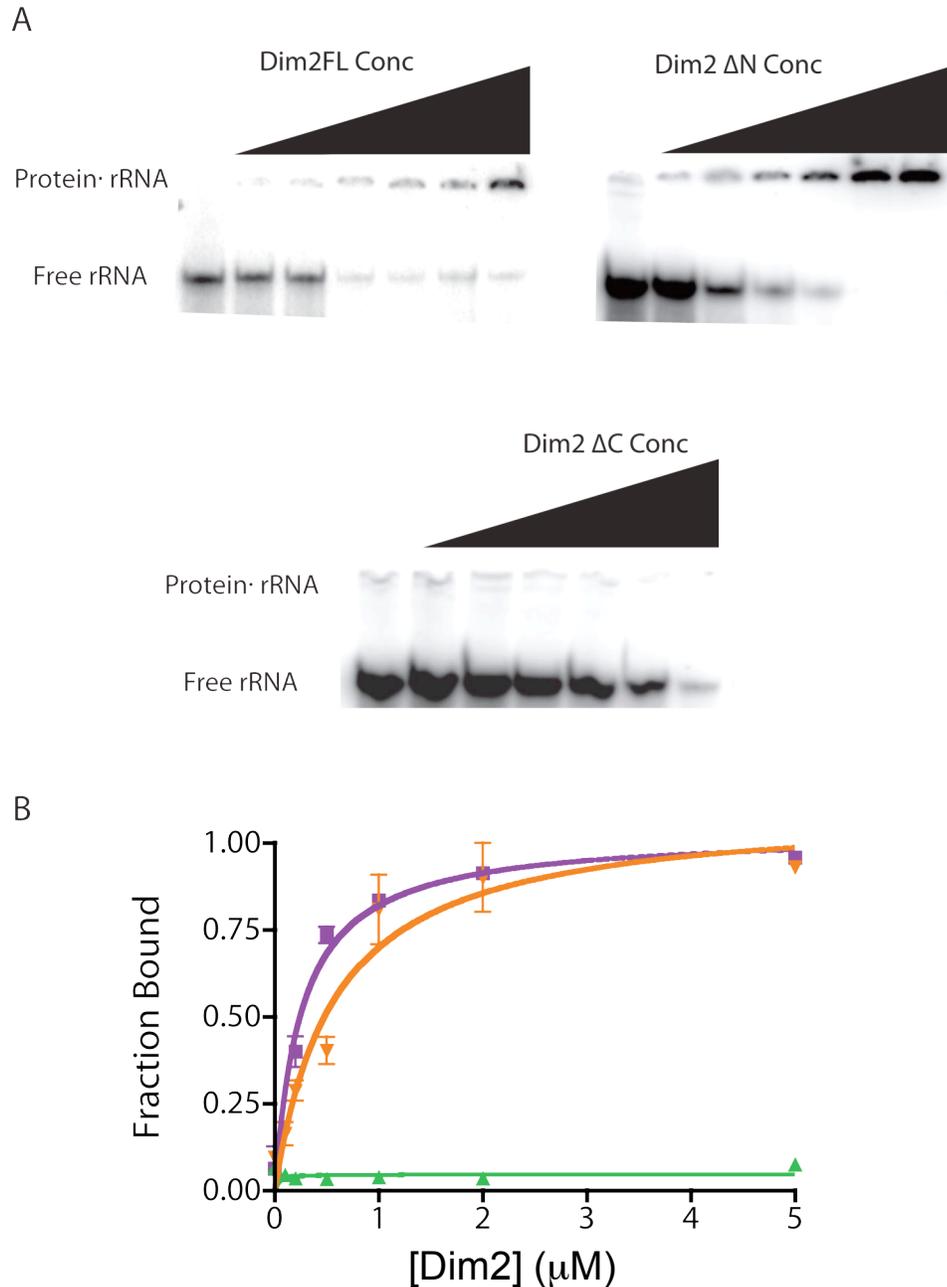


Figure 15. C-terminal KH domain of Dim2 binds rRNA. A) Gel-shift assays show full length Dim2 and N-terminal KH truncation Dim2 bind rRNA analog transcripts. Deletion of the C-terminal KH domain abrogates binding to rRNA transcripts. B) Quantification of gel shift assays demonstrate similar binding affinities of full length Dim2 (in orange) and Δ N Dim2 (in purple) for rRNA. Dim2 Δ C (in green) does not bind rRNA.

N-terminal KH domain is not essential, but Dim2 dimerization is functional

We created a yeast strain that would allow us to study the effect of this N-terminal KH domain *in vivo*. Since Dim2 is essential, we placed the genomic

Dim2 gene under galactose control, which allowed us to shut off expression of Dim2 by growth on glucose. As shown in Figure 16, when plated on glucose, this yeast strain cannot grow in the absence of Dim2, verifying that Dim2 is essential. Growth on glucose is completely restored when cells are transformed with a vector containing Dim2 on a plasmid. As demonstrated in a serial dilution, Figure 16 shows that when complemented with a plasmid containing the N-terminal KH deletion, cells are still viable, demonstrating that the N-terminal KH domain is not essential. Additionally, when complemented with a plasmid containing the WK/A mutation, which disrupts Nob1 binding *in vitro*, cells remain viable, indicating that this double mutant does not completely disrupt the function of Dim2 completely *in*

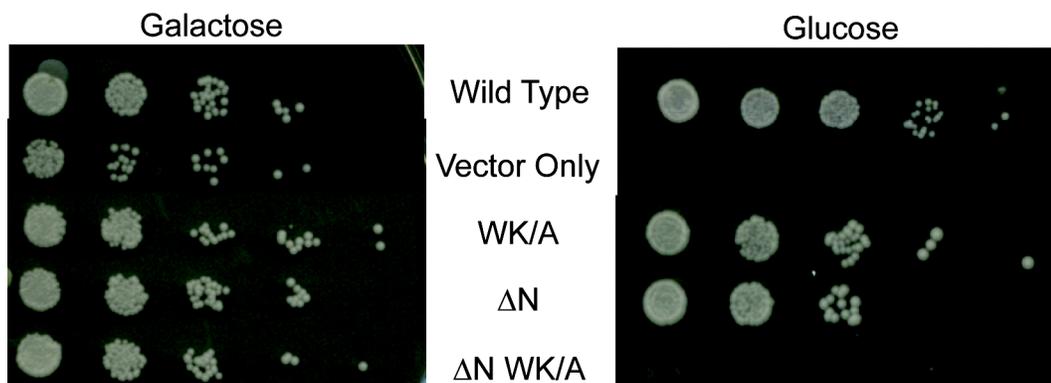


Figure 16. Dim2 is essential; N-terminal KH domain is not essential. A galactose-inducible Dim2 strain of yeast shows that cells cannot grow in the absence of Dim2. Deletion of the N-terminal KH domain minimally affects viability unless coupled with the WK/A mutation, which has no effect on growth when present in full-length Dim2.

vivo. However, when cells are complemented with plasmids containing the combination of the WK/A mutation and the N-terminal KH deletion, viability is lost, indicating a functional interaction between molecules in a Dim2 dimer.

Discussion

Dim2 contains three KH domains.

KH domains are well-characterized RNA binding domains, in which a three-stranded antiparallel β -sheet packs against three α -helices (Valverde *et al.*, 2008). The RNA binding surface is formed by a conserved GxxG loop, the first two α -helices, and the second β -sheet. Previous research has shown that Dim2 contains a C-terminal KH domain that is conserved (Vanrobays *et al.*, 2008). We have presented evidence that Dim2 contains two additional KH domains. The N-terminal KH domain contains all the conserved secondary structure elements as well as conserved hydrophobic residues that typically stabilize KH folds as well as the canonical GxxG loop. We have shown that this domain is involved in Dim2 dimerization; summarized in Figure 17. Previous structural work with proteins containing multiple KH-domains has shown that in several cases two KH domains can interact with each other. This interaction involves residues that are on the opposite side of the protein compared to the RNA binding surface, and there is no evidence that dimerization of KH domains has functional significance (for review see: Valverde *et al.*, 2007). Our research has shown that when the N-terminal KH domain of Dim2 is deleted, the protein elutes as a monomer from size-exclusion chromatography and that this monomer can still bind Nob1, indicating that dimerization is not necessary to bind Nob1.

We designate the central domain as a divergent KH domain because it contains all the conserved secondary structure elements as well as conserved hydrophobic residues, yet it does not contain the GxxG loop, which is instead

replaced with the sequence NSWT. Our *in vitro* assays show that the central diverged KH domain of Dim2 provides the interface for interaction with Nob1.

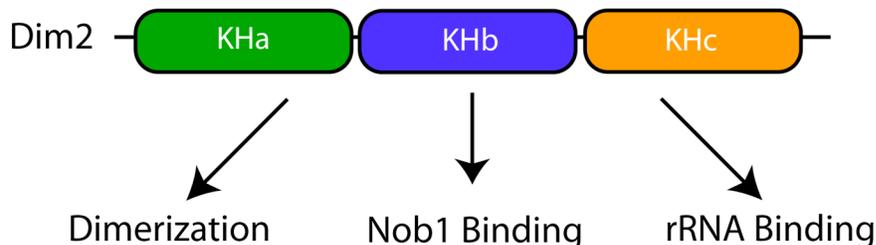


Figure 17. Functions of Dim2 KH domains.

A divergent KH domain is involved in a protein-protein interaction.

In vivo pull downs and a yeast 2-hybrid screen suggested an interaction between Dim2 and Nob1 (Tone & Toh-E, 2002). However, considering the large number of false positives usually obtained through yeast 2-hybrid screens and knowing that Dim2 and Nob1 bind to the same ribosome assembly intermediates, we wanted to test whether there was a direct interaction between Nob1 and Dim2 using our recombinant protein system. The *in vitro* pull-down experiments shown in this chapter verify a direct interaction, which we were able to map to the middle divergent KH domain of Dim2 (summarized in Figure 17). We confirmed that this domain was responsible for binding Nob1 by mutating the tryptophan found in the NSWT loop that modeled to the site of a conserved GxxG loop in canonical KH domains. Our results show this mutation disrupts the Dim2-Nob1 interaction.

Previous research has shown that KH domains can interact via an interaction site on the opposite side of the protein from the RNA binding surface (Du *et al.*, 2007; Git & Standart, 2002; Lin *et al.*, 1997; Ramos *et al.*, 2002), and

while we have implicated the NSWT loop in Nob1 binding, it remains to be seen whether this protein-protein interaction uses the same KH-domain surface interface as RNA binding or the “back-side” of the molecule, as seen with dimerization. It has been suggested before that individual KH domains in proteins with multiple KH domains interact with proteins via KH domain interactions (reviewed in Valverde *et al.*, 2007). For example, the last two KH-domains in human vigilin have been shown to interact with a histone-methyltransferase even in the absence of RNA, suggesting strongly that at least one of these KH domains is a protein-interacting module, although the mode of interaction (via the RNA binding surface or other surfaces) is not clear. Interestingly, not all 15 KH domains in vigilin contain the canonical GxxG loop. However, the two terminal KH domains contain conserved GxxG loops indicating that the ability of KH domains to bind protein or RNA cannot be predicted based on the presence or absence of a divergent or conserved loop.

Evidence of functional significance of KH dimerization

Previous evidence suggested KH domains could interact, but there was no evidence as to a functional significance of potential dimerization of KH domains. Here we have shown a functional significance in dimerization of Dim2 through an N-terminal KH domain as evidenced by the double mutation in the galactose-inducible Dim2 yeast strain (detailed in Figure 16). Disruption of Dim2 dimerization alone is not lethal. However, coupled with mutations in the middle KH domain of Dim2 loss of dimerization is lethal. Considering Dim2 binds rRNA through the C-terminal KH domain and binds another assembly factor, Nob1

through the middle KH domain, this data suggests that both monomers in a Dim2 dimer are involved in proper Dim2 function. Research has also shown that Dim2 interacts with an additional assembly factor, Rio2 (Campbell & Karbstein, 2010), suggesting that Dim2 could interact with additional assembly factors at various stages in ribosome assembly.

Materials and Methods

Protein expression plasmids: Dim2 open reading frame, N-terminal, and C-terminal deletion constructs were cloned into pSV272 for protein expression in (*E. coli*) Rosetta competent cells using oligonucleotides listed in Table 1.

Mutagenesis: Dim2 mutants were generated using the Quikchange protocol. See Table 1 for mutagenesis primers used in cloning.

Expression and Purification of Dim2: His-MBP-tagged Dim2 plasmid DNA (pSV272; wild type, mutants, and truncations) was freshly transformed into Rosetta cells. Protein expression was carried out as previously described (Lamanna & Karbstein, 2009). Cells were lysed in 50 mM NaPO₄ (pH 8.0), 150 mM NaCl, and 0.5 mM PMSF and purified via Ni-NTA chromatography using 1 ml HisTrap Columns (GE Healthcare). Ni eluate fractions containing Dim2 were pooled and dialyzed 3 h (or overnight) into 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM DTT (Buffer A) at 4 °C. Dialyzed protein was loaded onto a MonoS column equilibrated in Buffer A and eluted in a salt gradient to a final concentration of 40% of Buffer B (50 mM Tris (pH 8.0), 2 M NaCl, 1 mM DTT) over 12 column

volumes. Fractions containing Dim2 were pooled and concentrated and further purified over a Superdex200 column in 50 mM HEPES (pH 8.0), 200 mM NaCl, 1 mM DTT. Dim2-containing fractions were pooled and concentrated and flash frozen and stored at -80 °C. Protein concentration was determined with a nanodrop spectrophotometer using Beer's law.

Dim2 mutant proteins were purified using the same protocol as the wild type protein with the exception that the mutant proteins bound to the MonoQ ion-exchange column.

Transcriptions: rDNA fragments were cloned into pUC19 between XmaI and PstI sites as described (Lamanna & Karbstein, 2009). 50 mL overnight cultures were grown in LB medium with Ampicillin and plasmid DNA isolated using a maxiprep kit (Qiagen). Plasmid DNA was digested with BtsI (NEB), phenol: chloroform extracted and resuspended in 200 µL of water. Transcriptions were carried out as described (Lamanna & Karbstein, 2009) using the entire digested rDNA template in a final volume of 5 µL. The reaction was terminated by adding 0.5 µL of 0.5 M EDTA (pH 8.0), and RNA was precipitated overnight at -20°C by adding 0.55 µL of 3 M sodium acetate and 16.5 µL ethanol. RNA was purified using an RNA Maxi Kit (Qiagen). To obtain internally-labeled transcripts reactions were carried out as previously described (Lamanna & Karbstein, 2009).

RNA Binding Assay: RNA was folded as described (Lamanna & Karbstein, 2009). Dim2 was pre-incubated at 30 °C for 15-20 minutes before RNA was

added to binding tubes. 10 μ l binding reactions were incubated at 30 °C for 2 hours and then immediately loaded onto an 8% Acrylamide/THEM (pH 8.0) gels (33 mM Tris, 67 mM HEPES, 1 mM EDTA, 10 mM MgCl₂) after addition of 2 μ l of RNA loading dye (0.1% xylene cyanol, 0.1% bromophenol blue, 45% glycerol). Gels were run at 250V for ~2 hours, dried and exposed to a phosphoscreen overnight. Signal was detected using Typhoon scanner and quantification was performed using ImageQuant software.

In vitro Pull Down Assays: 10 μ M recombinant MBP-tagged Dim2 (or MBP-tagged Nob1) was incubated with 10 μ M or 40 μ M untagged Nob1 (or untagged Dim2) for 30 minutes at room temperature (in size-exclusion buffer from purification). Pre-equilibrated amylose resin was added to protein mixture and incubated on a shaker for 30 minutes at room temperature. The proteins-amylose resin mixture was applied to a disposable column and the flow through was collected by centrifugation for a couple of seconds. The column was washed with 1ml of buffer and centrifuged for a couple of seconds to collect all residual buffer. Bound proteins were eluted with a 50 mM maltose solution (50 mM maltose in size-exclusion buffer) and analyzed via SDS-PAGE.

Dim2 Modeling: The open reading frame sequence for *S. cerevisiae* Dim2 (YOR145C; Chromosome XV: 606172 to 605348) was submitted to two protein structure homology modeling servers, SwissModel (<http://swissmodel.expasy.org/> Arnold, *et al.*, 2006; Bordoli *et al.*, 2009; Kiefer, *et*

et al., 2009) and 3Djigsaw (<http://bmm.cancerresearchuk.org/~3djigsaw/> Bates, *et al.*, 2001). Both servers modelled the c-terminal two-thirds of the sequence to the crystal structure of Dim2p from *Pyrococcus horikoshii* OT3 (PDB entrant 2E3U), an archael homolog of *S. cerevisiae* Dim2 (Jia, *et al.*, 2007). The models were analyzed using Pymol software.

Secondary Structure Predictions: The open reading frame sequence for *S. cerevisiae* Dim2 was submitted to the secondary structure predictions programs Jpred (<http://www.compbio.dundee.ac.uk/~www-jpred/index.html> Cole, *et al.*, 2008), SMART (<http://smart.embl-heidelberg.de/> Letunic, *et al.*, 2009; Schultz, *et al.*, 1998) and Predictprotein (<http://www.predictprotein.org/> Rost, *et al.*, 2004).

Yeast Strains and Yeast Expression Plasmids: Yeast strain YKK192, created from the Open Biosystems Nob1-TAP strain, has a galactose-inducible promoter inserted in front of the Dim2 open reading frame. It was generated using PCR-based recombination (Longtine *et al.*, 1998), and correct insertion was verified by PCR and Western analysis. The Dim2 open reading frame, including an N-terminal HA-tag, was cloned into pRS416TEF using oligonucleotides listed in Table 1.

Table 1: Primers Used for Cloning Dim2

Primers for Cloning into pSV272	
FLwt 5' (Sfo1)	GATCGAGGCGCCATGGTTGCGCCTA
FLwt 3' (HindIII)	TCAGACAAGCTT TTAGTAGCGTTCT TTAATCTAG
N-terminal Deletion 5' (Sfo1)	GTAGTAGGCGCCACACAGGGTAACA AAATAAAATTTGAATCGAG
C-terminal Deletion 3' (HindIII)	CAAGTAAAGCTTTCAATCTAGAATCA ATAGTGAGATAGAATCG

Mutagenesis Primers	
Dim2 WK/A	CACCCTTGAGGAACAGTGCTAC GGCAATTTATCCTCCTTTGG
Primers for Cloning into pSV272	
FLwt 5' (Sfo1)	GATCGAGGCGCCATGGTTGCGCCTA
FLwt 3' (HindIII)	TCAGACAAGCTT TTAGTAGCGTTCT TTAATCTAG
N-terminal Deletion 5' (Sfo1)	GTAGTAGGCGCCACACAGGGTAACA AAATAAAATTTGAATCGAG
C-terminal Deletion 3' (HindIII)	CAAGTAAAGCTTTCAATCTAGAATCA ATAGTGAGATAGAATCG

Mutagenesis Primers	
Dim2 WK/A	CACCCTTGAGGAACAGTGCTAC GGCAATTTATCCTCCTTTGG

References

- Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics (Oxford, England)*, *22*(2), 195-201.
doi:10.1093/bioinformatics/bti770
- Backe, P. H., Messias, A. C., Ravelli, R. B., Sattler, M., & Cusack, S. (2005). X-ray crystallographic and NMR studies of the third KH domain of hnRNP K in complex with single-stranded nucleic acids. *Structure (London, England : 1993)*, *13*(7), 1055-1067. doi:10.1016/j.str.2005.04.008
- Bates, P. A., Kelley, L. A., MacCallum, R. M., & Sternberg, M. J. (2001). Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. *Proteins, Suppl 5*, 39-46.
- Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J., & Schwede, T. (2009). Protein structure homology modeling using SWISS-MODEL workspace. *Nature Protocols*, *4*(1), 1-13. doi:10.1038/nprot.2008.197
- Braddock, D. T., Baber, J. L., Levens, D., & Clore, G. M. (2002). Molecular basis of sequence-specific single-stranded DNA recognition by KH domains: Solution structure of a complex between hnRNP K KH3 and single-stranded DNA. *The EMBO Journal*, *21*(13), 3476-3485. doi:10.1093/emboj/cdf352
- Chmiel, N. H., Rio, D. C., & Doudna, J. A. (2006). Distinct contributions of KH domains to substrate binding affinity of drosophila P-element somatic inhibitor protein. *RNA (New York, N.Y.)*, *12*(2), 283-291.
doi:10.1261/rna.2175706

- Clery, A., Blatter, M., & Allain, F. H. (2008). RNA recognition motifs: Boring? not quite. *Current Opinion in Structural Biology*, *18*(3), 290-298.
doi:10.1016/j.sbi.2008.04.002
- Cole, C., Barber, J. D., & Barton, G. J. (2008). The jpred 3 secondary structure prediction server. *Nucleic Acids Research*, *36*(Web Server issue), W197-201.
doi:10.1093/nar/gkn238
- Du, Z., Fenn, S., Tjhen, R., & James, T. L. (2008). Structure of a construct of a human poly(C)-binding protein containing the first and second KH domains reveals insights into its regulatory mechanisms. *The Journal of Biological Chemistry*, *283*(42), 28757-28766. doi:10.1074/jbc.M803046200
- Du, Z., Lee, J. K., Fenn, S., Tjhen, R., Stroud, R. M., & James, T. L. (2007). X-ray crystallographic and NMR studies of protein-protein and protein-nucleic acid interactions involving the KH domains from human poly(C)-binding protein-2. *RNA (New York, N.Y.)*, *13*(7), 1043-1051. doi:10.1261/rna.410107
- Du, Z., Lee, J. K., Tjhen, R., Li, S., Pan, H., Stroud, R. M., & James, T. L. (2005). Crystal structure of the first KH domain of human poly(C)-binding protein-2 in complex with a C-rich strand of human telomeric DNA at 1.7 Å. *The Journal of Biological Chemistry*, *280*(46), 38823-38830. doi:10.1074/jbc.M508183200
- Fatica, A., & Tollervey, D. (2002). Making ribosomes. *Current Opinion in Cell Biology*, *14*(3), 313-318.
- Ferreira-Cerca, S., Poll, G., Gleizes, P. E., Tschochner, H., & Milkereit, P. (2005). Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S

- rRNA and ribosome function. *Molecular Cell*, 20(2), 263-275.
doi:10.1016/j.molcel.2005.09.005
- Ferreira-Cerca, S., Poll, G., Kuhn, H., Neueder, A., Jakob, S., Tschochner, H., & Milkereit, P. (2007). Analysis of the in vivo assembly pathway of eukaryotic 40S ribosomal proteins. *Molecular Cell*, 28(3), 446-457.
doi:10.1016/j.molcel.2007.09.029
- Flynn, R. L., & Zou, L. (2010). Oligonucleotide/oligosaccharide-binding fold proteins: A growing family of genome guardians. *Critical Reviews in Biochemistry and Molecular Biology*, 45(4), 266-275.
doi:10.3109/10409238.2010.488216
- Fromont-Racine, M., Senger, B., Saveanu, C., & Fasiolo, F. (2003). Ribosome assembly in eukaryotes. *Gene*, 313, 17-42.
- Garcia-Mayoral, M. F., Diaz-Moreno, I., Hollingworth, D., & Ramos, A. (2008). The sequence selectivity of KSRP explains its flexibility in the recognition of the RNA targets. *Nucleic Acids Research*, 36(16), 5290-5296.
doi:10.1093/nar/gkn509
- Git, A., & Standart, N. (2002). The KH domains of xenopus Vg1RBP mediate RNA binding and self-association. *RNA (New York, N.Y.)*, 8(10), 1319-1333.
- Gopal, B., Haire, L. F., Gamblin, S. J., Dodson, E. J., Lane, A. N., Papavinasasundaram, K. G., Colston, M. J., & Dodson, G. (2001). Crystal structure of the transcription elongation/anti-termination factor NusA from mycobacterium tuberculosis at 1.7 Å resolution. *Journal of Molecular Biology*, 314(5), 1087-1095. doi:10.1006/jmbi.2000.5144

- Grishin, N. V. (2001). KH domain: One motif, two folds. *Nucleic Acids Research*, 29(3), 638-643.
- Jensen, K. B., Musunuru, K., Lewis, H. A., Burley, S. K., & Darnell, R. B. (2000). The tetranucleotide UCAY directs the specific recognition of RNA by the nova K-homology 3 domain. *Proceedings of the National Academy of Sciences of the United States of America*, 97(11), 5740-5745.
doi:10.1073/pnas.090553997
- Jia, M. Z., Ohtsuka, J., Lee, W. C., Nagata, K., & Tanokura, M. (2007). Crystal structure of Dim2p: A preribosomal RNA processing factor, from pyrococcus horikoshii OT3 at 2.30 Å. *Proteins*, 69(2), 428-432. doi:10.1002/prot.21381
- Kiefer, F., Arnold, K., Kunzli, M., Bordoli, L., & Schwede, T. (2009). The SWISS-MODEL repository and associated resources. *Nucleic Acids Research*, 37(Database issue), D387-92. doi:10.1093/nar/gkn750
- Klug, A. (2010). The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annual Review of Biochemistry*, 79, 213-231. doi:10.1146/annurev-biochem-010909-095056
- Lamanna, A. C., & Karbstein, K. (2009a). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings of the National Academy of Sciences of the United States of America*, 106(34), 14259-14264. doi:10.1073/pnas.0905403106
- Lamanna, A. C., & Karbstein, K. (2009b). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings*

- of the National Academy of Sciences of the United States of America*,
106(34), 14259-14264. doi:10.1073/pnas.0905403106
- Letunic, I., Doerks, T., & Bork, P. (2009). SMART 6: Recent updates and new developments. *Nucleic Acids Research*, 37(Database issue), D229-32. doi:10.1093/nar/gkn808
- Lin, Q., Taylor, S. J., & Shalloway, D. (1997). Specificity and determinants of Sam68 RNA binding. implications for the biological function of K homology domains. *The Journal of Biological Chemistry*, 272(43), 27274-27280.
- Maris, C., Dominguez, C., & Allain, F. H. (2005). The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. *The FEBS Journal*, 272(9), 2118-2131. doi:10.1111/j.1742-4658.2005.04653.x
- Musunuru, K., & Darnell, R. B. (2004). Determination and augmentation of RNA sequence specificity of the nova K-homology domains. *Nucleic Acids Research*, 32(16), 4852-4861. doi:10.1093/nar/gkh799
- Osheim, Y. N., French, S. L., Keck, K. M., Champion, E. A., Spasov, K., Dragon, F., Baserga, S. J., & Beyer, A. L. (2004). Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to being cleaved from nascent transcripts in *saccharomyces cerevisiae*. *Molecular Cell*, 16(6), 943-954. doi:10.1016/j.molcel.2004.11.031
- Ramos, A., Hollingworth, D., Major, S. A., Adinolfi, S., Kelly, G., Muskett, F. W., & Pastore, A. (2002). Role of dimerization in KH/RNA complexes: The example of nova KH3. *Biochemistry*, 41(13), 4193-4201.

- Rost, B., Yachdav, G., & Liu, J. (2004). The PredictProtein server. *Nucleic Acids Research*, 32(Web Server issue), W321-6. doi:10.1093/nar/gkh377
- Schultz, J., Milpetz, F., Bork, P., & Ponting, C. P. (1998). SMART, a simple modular architecture research tool: Identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America*, 95(11), 5857-5864.
- Siomi, H., Matunis, M. J., Michael, W. M., & Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Research*, 21(5), 1193-1198.
- Siomi, H., Siomi, M. C., Nussbaum, R. L., & Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. *Cell*, 74(2), 291-298.
- Tone, Y., & Toh-E, A. (2002). Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *Saccharomyces cerevisiae*. *Genes & Development*, 16(24), 3142-3157. doi:10.1101/gad.1025602
- Valverde, R., Edwards, L., & Regan, L. (2008). Structure and function of KH domains. *The FEBS Journal*, 275(11), 2712-2726. doi:10.1111/j.1742-4658.2008.06411.x
- Valverde, R., Pozdnyakova, I., Kajander, T., Venkatraman, J., & Regan, L. (2007). Fragile X mental retardation syndrome: Structure of the KH1-KH2 domains of fragile X mental retardation protein. *Structure (London, England : 1993)*, 15(9), 1090-1098. doi:10.1016/j.str.2007.06.022

Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., & Lafontaine, D. L. (2004).

Dim2p, a KH-domain protein required for small ribosomal subunit synthesis.

RNA (New York, N.Y.), 10(4), 645-656.

Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., &

Lafontaine, D. L. (2008a). TOR regulates the subcellular distribution of DIM2,

a KH domain protein required for cotranscriptional ribosome assembly and

pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.

doi:10.1261/rna.1176708

Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., &

Lafontaine, D. L. (2008b). TOR regulates the subcellular distribution of DIM2,

a KH domain protein required for cotranscriptional ribosome assembly and

pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.

doi:10.1261/rna.1176708

Wen, W. L., Stevenson, A. L., Wang, C. Y., Chen, H. J., Kearsey, S. E., Norbury,

C. J., Watt, S., Bahler, J., & Wang, S. W. (2010). Vgl1, a multi-KH domain

protein, is a novel component of the fission yeast stress granules required for

cell survival under thermal stress. *Nucleic Acids Research*,

doi:10.1093/nar/gkq555

CHAPTER III

Dim2 Roles in Ribosome Assembly

Introduction

Ribosome assembly in eukaryotes is a complex and highly regulated process, which consumes much of the energy of an actively growing cell (Warner, 1999; Warner, *et al.*, 2001). Ribosome assembly begins with the transcription of four ribosomal RNAs (rRNAs), three of which are produced from a single transcript that is cotranscriptionally processed to produce premature 18S, 5.8S and 25S rRNAs. Cotranscriptional processing includes extensive 2'-OH methylations, pseudouridylations (Kos & Tollervey, 2010), and cleavages to produce the premature small and large ribosomal subunits (Osheim *et al.*, 2004; Figure 1a), as well as incorporation of ribosomal proteins. Data in the literature indicate that 18S rRNA/small subunit processing is initiated by a non-essential cleavage step in the region 5' to 18S rRNA (5'-ETS), termed A₀, followed by the essential cleavage steps at sites A₁ (to generate the mature 5'-end of the 18S rRNA) and A₂ (to separate the premature small subunit from the large subunit precursor) (Venema & Tollervey, 1999). Maturation of the small subunit includes export into the cytoplasm (Schafer, *et al.*, 2003; Thomas & Kutay, 2003; Tschochner & Hurt, 2003) and Nob1 cleavage of the 18S rRNA at site D to produce the mature 3'-end of the rRNA (Figure 18; Fatica, *et al.*, 2004; Lamanna & Karbstein, 2009b). The process of producing mature, functional ribosomal

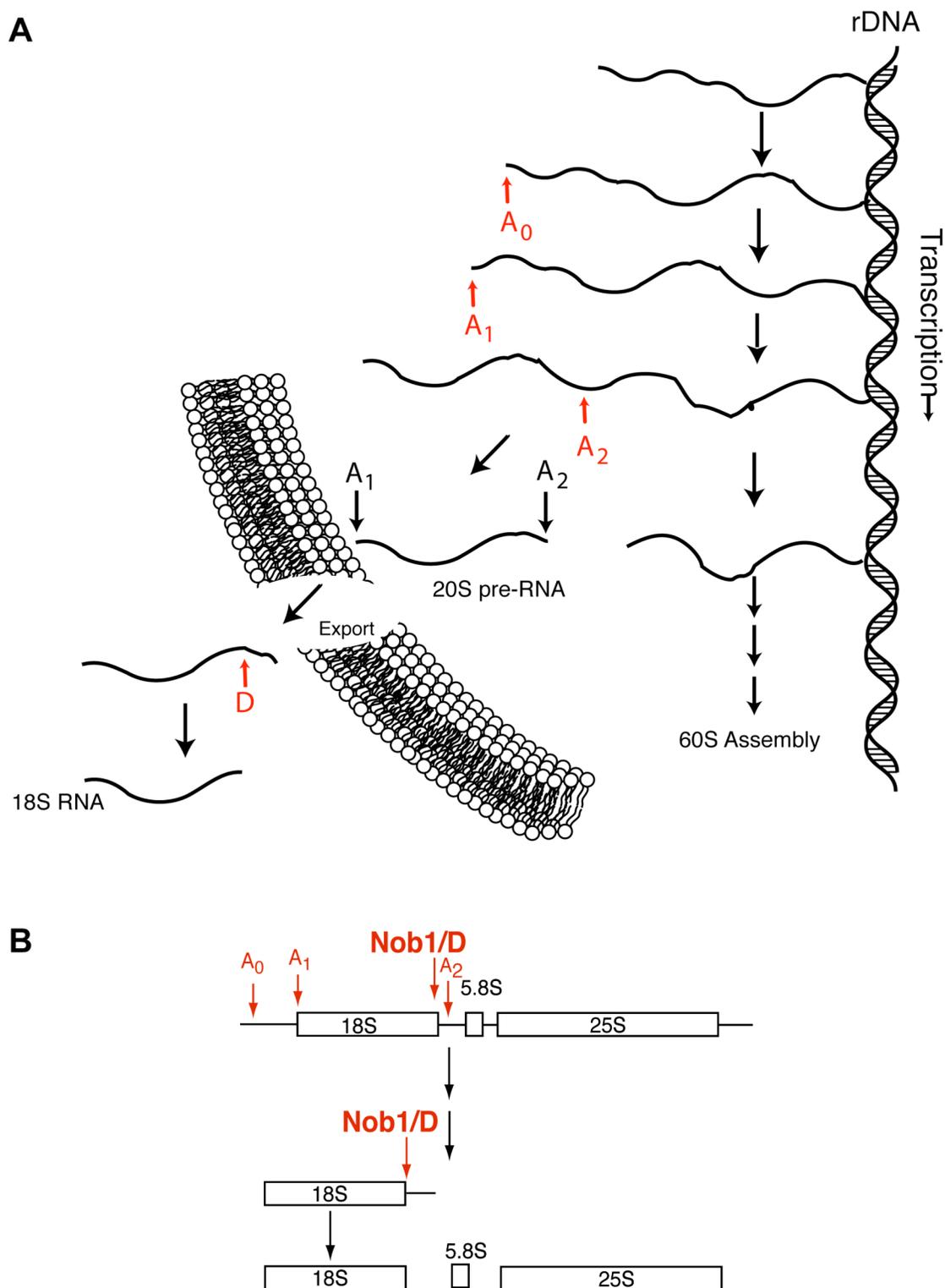


Figure 18. Schematic of ribosomal RNA transcription and processing occurs simultaneously. A) Transcription of rRNA begins in the nucleolus where initial processing occurs cotranscriptionally. Following A_2 cleavage, the small subunit precursor is exported in to the cytoplasm where maturation takes place. B) Nob1 is the D-site endonuclease responsible for cytoplasmic maturation of the small subunit rRNA.

subunits is a complex assembly process that requires approximately 200 essential assembly factors which transiently associate to pre-ribosomal complexes (Bernstein *et al.*, 2004; Fromont-Racine *et al.*, 2003). Despite their necessity, the specific role of most of the assembly factors in ribosome biogenesis remains unknown. Since the role of enzymatic proteins can be dissected relatively easily, nearly all rRNA modifying enzymes and nucleolytic enzymes have been identified. However, the roles of the majority of essential assembly factors are harder to determine, as most of these proteins only contain RNA binding or protein-protein interaction motifs.

To tackle this problem, Tollervy and colleagues have used a crosslinking technique to elegantly map the RNA binding sites of several assembly factors (Granneman *et al.*, 2010). However this technique has not produced crosslinks between all assembly factors and RNA. Dim2 is one such protein.

In the previous chapter, we have described an interaction between Dim2 and the D-site cleavage endonuclease Nob1. We have shown that this protein-protein interaction involves the central KH-like diverged KH domain of Dim2. Here, we use mutations in this diverged KH domain to map and disrupt the Nob1 interaction and dissect the effect of Dim2 on ribosome assembly. The data provide evidence for a Dim2 role in recruiting Nob1 to pre-ribosomes and regulating this endonuclease for D-site cleavage.

Results

In the previous chapter we have shown that there is a direct interaction between Dim2 and Nob1 and that the central, diverged KH domain in Dim2 mediates this interaction. We wanted to further dissect the interaction between Dim2 and Nob1 to determine what role this interaction plays in ribosome assembly.

Dim2 Model Suggests Residues Involved In Nob1 Interaction.

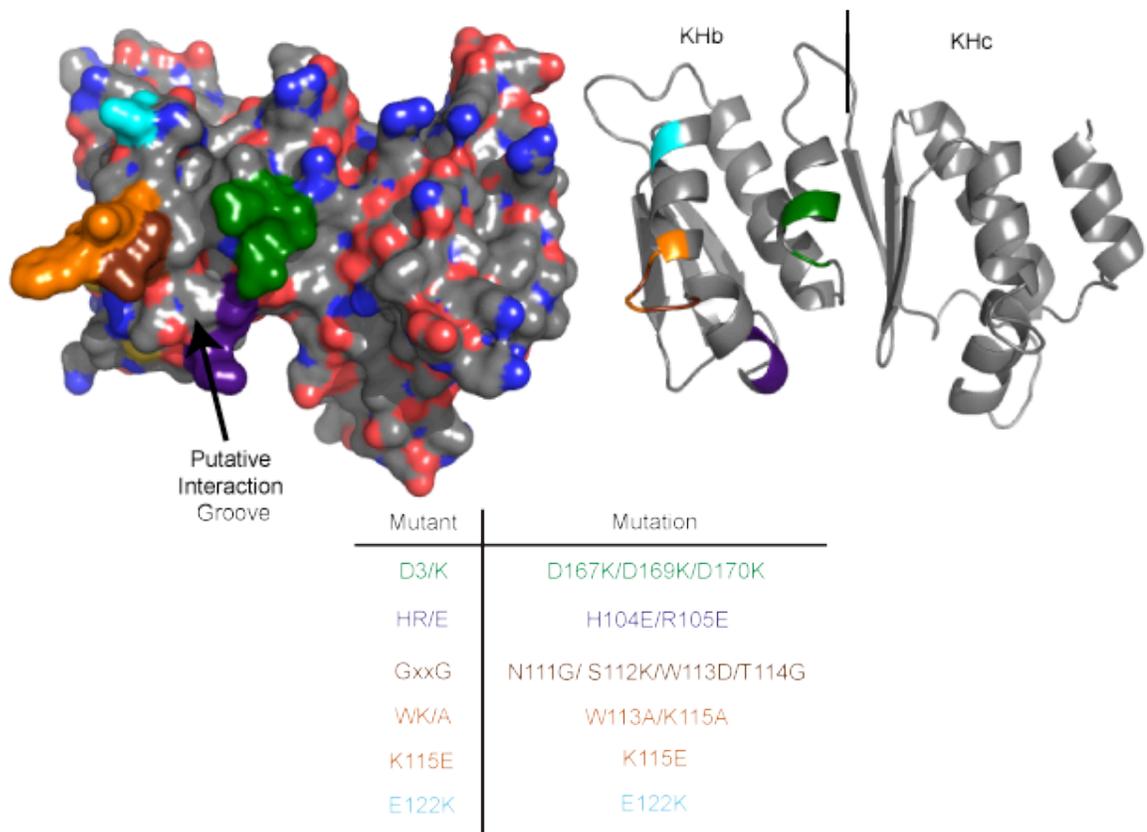


Figure 19. Model of Dim2 Suggest Binding Interface. The sequence of *S. cerevesiae* Dim2 modelled to an archael Dim2. The surface representation of the model, shown on the left, suggested a surface groove along the middle KH domain that could bind Nob1. Highly conserved, surface-exposed charge residues were mutated to the opposite charge, shown below. The ribbon diagram of the model is shown on the right to illustrate the mutations are in the KH domain.

The model for the central and C-terminal domains of Dim2 suggests a surface groove in the central diverged KH domain. This surface groove is adjacent to the GxxG loop, already suggested to be involved in Nob1 binding (Chapter II) and is lined with several highly conserved, charged residues. Because of the conservation of these surface exposed residues, we hypothesize that they could be involved in Nob1 binding (Figure 19). We mutated several of these residues to test whether these residues could be involved in Nob1 binding. We made a triple mutant by mutating the cluster of aspartic acid residues (shown in green; residues 167, 169, and 170; referred to as D3/K) to lysines, a double mutant by mutating the tandem basic residues, histidine 104 and arginine 105, to glutamates (shown in purple; referred to as HR/E) and the point mutants E122K, and K115E. We also substituted the NSWT sequence with the GxxG sequence found in the C-terminal conserved KH domain (referred to as GxxG). We were able to use these mutations to study the function of this specific protein-protein interaction in ribosome assembly.

Mutations in Dim2 are lethal.

We created a yeast strain that would allow us to study the effect of these Dim2 mutations *in vivo*. Since Dim2 is essential (Senapin *et al.*, 2003; Vanrobays *et al.*, 2004) we placed the genomic Dim2 gene under galactose control, which allowed us to shut off expression of Dim2 by growth of glucose. As shown in Figure 20, when plated on glucose these cells cannot grow when Dim2 is depleted, verifying that it is essential. Viability is restored when cells are

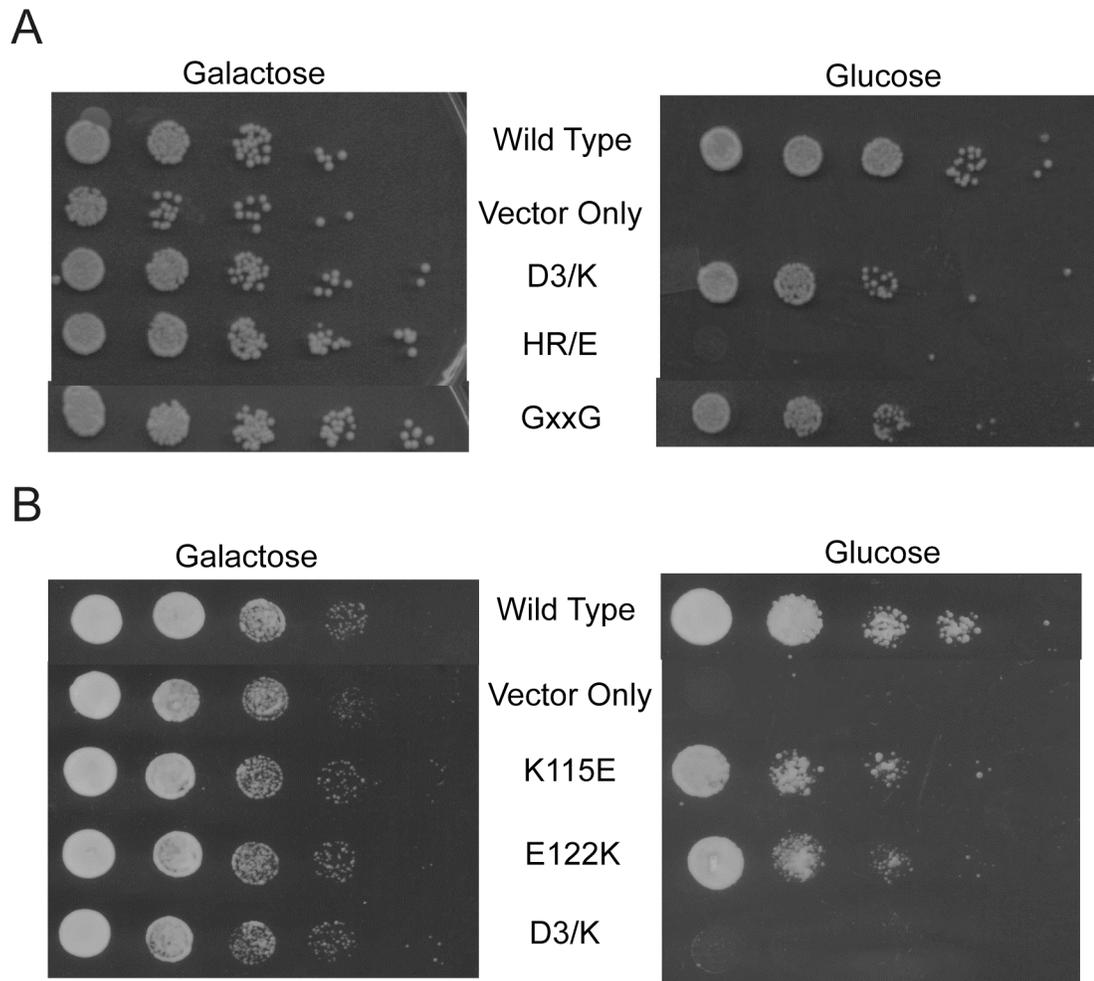


Figure 20. Galactose-inducible Dim2 strain shows essential residues. A) Dim2 constitutively expressed under a strong TEF promoter shows growth defects with the D3/K, HR/E, and GxxG mutants. B) Even when constitutively expressed under the weak Cyc1 promoter, mutants K115E and E122K showed minimal growth defects.

supplemented with a vector containing wild type, full-length Dim2. To determine whether or not these surface exposed mutations in Dim2 would affect viability, we supplemented the galactose-inducible strain with plasmids encoding the mutations. As shown in Figure 20, the HR/E mutant did not restore growth at all, while the GxxG and D3/K mutants partially restored growth. The E122K and K115E mutants completely restored growth. Important to note, western blotting

showed that the mutations did not disrupt the stability of the proteins as determined by western blots (data not shown). Since we were able to determine that these residues are important for the function of Dim2, we wanted to use the *in vitro* pull-down system to determine if these residues are directly involved in binding Nob1.

Mutations in Dim2 Disrupt Nob1 Binding In vitro.

We were able to overexpress and purify MBP-N-terminal fusions of the D3/K, HR/E, and GxxG mutants as described below.

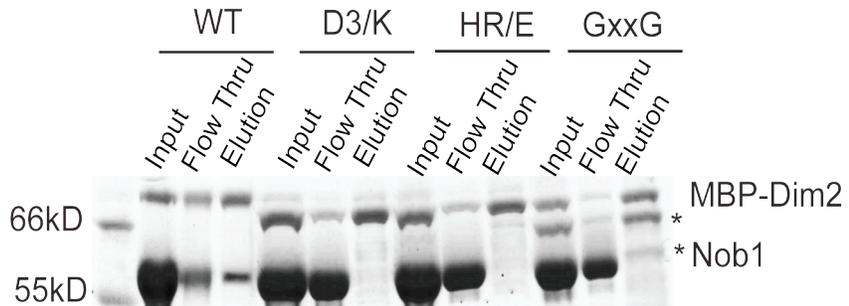


Figure 21. Dim2 Mutations Disrupt Nob1 Binding *In vitro*. Nob1 interacts with MBP-Dim2 in pull-down assays. This interaction is disrupted in D3/K, HR/E, and GxxG mutations in Dim2. Asterisks mark proteolysis products in GxxG as confirmed by western blotting. These proteolysis bands cross-reacted with a Dim2 antibody and not to a Nob1 antibody.

The mutant Dim2 proteins were incubated with untagged Nob1 as described for the wild type Dim2 pull-down experiments. As shown in Figure 21, the D3/K, HR/E, and GxxG mutations in Dim2 disrupt the *in vitro* interaction with Nob1 in the pull-down assay. Also shown in Figure 21, very little full-length Dim2 protein was obtained for the three mutants. Since the mutant proteins have an N-terminal MBP-fusion (used to bind the amylose resin in the *in vitro* assays), the proteolysis observed was at the C-terminus of the proteins. However, since we have shown that truncating the entire C-terminal KH domain does not disrupt

binding to Nob1 in our *in vitro* system (Chapter II), these proteolysis truncations do not appear to result in the loss of Nob1 binding.

Previous research has shown that removal of the C-terminal 39 residues of Dim2 abolishes binding to rRNA *in vitro* (Vanrobays *et al.*, 2008b). The RNA binding capacity of our mutants was tested using a gel-shift assay. Briefly, radioactively labeled rRNA transcripts were incubated with increasing amounts of

A



B

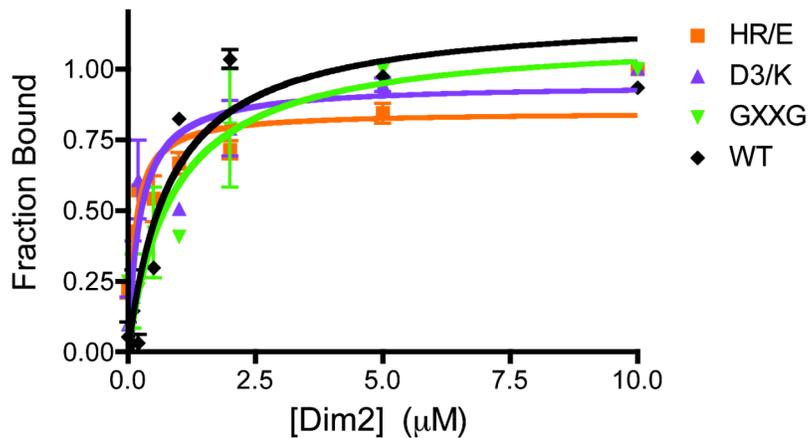


Figure 22. Dim2 mutations do not disrupt rRNA binding. A) Gel shift assays show the D3/K, HR/E, and GxxG mutants bind rRNA constructs, as does wild type Dim2¹. B) The fraction of RNA bound by Dim2 was quantified and fit with the Michaelis-Menten Equation to give $K_{1/2}$ values of 0.2 μM for D3/K and HR/E, 0.8 μM for wild type Dim2 and 1.5 μM for GxxG².

Dim2. The complexes were run on a native polyacrylamide gel and a decrease in electrophoretic mobility was observed with a larger protein-RNA complex when compared to RNA alone. Shown in Figure 22, all mutant, truncated proteins still bound RNA, indicating that the C-terminal truncations did not disrupt the RNA binding ability of the KH domain. The RNA binding activity of these mutant proteins also demonstrates that the truncated proteins are properly Dim2 but do disrupt Nob1 binding, supporting the idea that the central divergent KH domain of Dim2 interacts with Nob1.

Mutations in diverged KH domain result in 20S accumulation.

Previous research showed that deletion of Dim2 results in disruption of cleavage at site A_2 , and small ribosomal subunit formation, while deletion of Nob1 solely disrupts cytoplasmic cleavage at site D (Schafer *et al.*, 2003; Senapin *et al.*, 2003; Vanrobays *et al.*, 2008a). Because Nob1 deletion only disrupts a single step in ribosome assembly, deletion or mutations in Nob1 lead to an accumulation of 20S rRNA. We hypothesized that mutations in Dim2 that disrupt binding to Nob1 would also result in an accumulation of 20S if Dim2 were required for Nob1 to properly function in D-site cleavage. Additionally, if the mutations in Dim2 affect other processes, disruption in A_2 cleavage might be

1. Wild type Dim2 is purified over a MonoS ion-exchange chromatography column. However, the surface mutation in D3/K, HR/E, and GxxG changes the pI of the surface that interacts with the resin in the ion-exchange column. The mutants are purified using a MonoQ column (see Materials and Methods). This pI difference is also observed in the gel-shift assays where the wild-type protein-RNA complex does not run into the gel; however, the mutant protein-RNA complex does run into the gel. The two bound complexes, shown by the presence of two bands migrating into the gel, formed with the mutant proteins supports the idea of Dim2 functioning as a dimer (through the N-terminal KH domain).

2. The two-fold higher $K_{1/2}$ of GxxG can be explained by the proteolysis of the purified protein, as seen in Figure 3. This affects the concentration of GxxG that can bind rRNA but not RNA-binding capacity of GxxG.

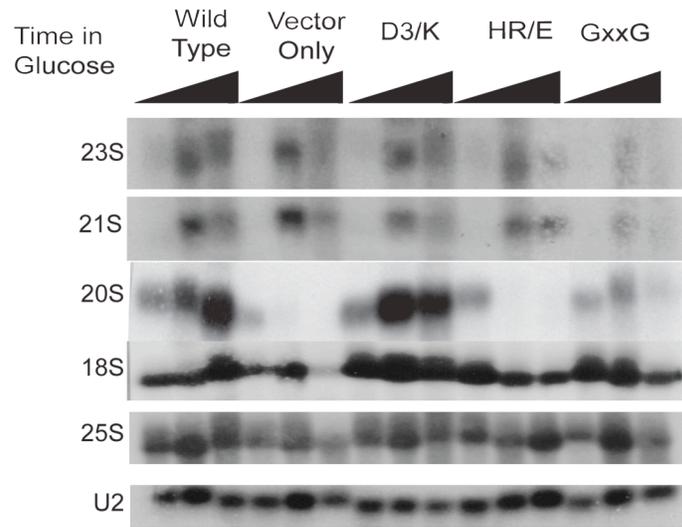
observed, similar to a Dim2 deletion phenotype.

Using the galactose-inducible Dim2 strain with transformed plasmids encoding wild type or mutant Dim2, we depleted endogenous Dim2 via growth in glucose for 0, 8 and 12 hrs, extracted total RNA from these cells and detected rRNA species by northern blotting. A probe directed against the spliceosomal U2 snRNA was used as a loading control. In Figure 23, as expected and consistent with prior publications, Northern blotting showed that depletion of Dim2 resulted in a complete loss of 20S rRNA and production of 21S and 23S rRNAs by 8 hours, indicating disruption of A_2 cleavage, and at least partial disruption of cleavages A_0 and A_1 .

As we hypothesized, when cells were depleted of endogenous wild type Dim2 and supplemented with the mutations in the diverged KH domain of Dim2, various phenotypes were observed. Interestingly, the D3/K mutant shows an accumulation of 20S rRNA consistent with a Dim2 requirement for Nob1 cleavage at site D. However, in the presence of the HR/E mutant, the pre-rRNA processing phenotype appears similar to that of the Dim2 depletion; while the GxxG mutant has an intermediate phenotype with a slight increase in 20S and a slightly increased production of 21S rRNA relative to growth in galactose (time point zero).

In addition to a Dim2 requirement for A_2 cleavage, these data show a requirement for proper D-site cleavage. Our data also shows that the interaction between the central diverged KH domain and Nob1 is required for D-site cleavage. This diverged KH domain is also required for A_2 cleavage, and since

A



B

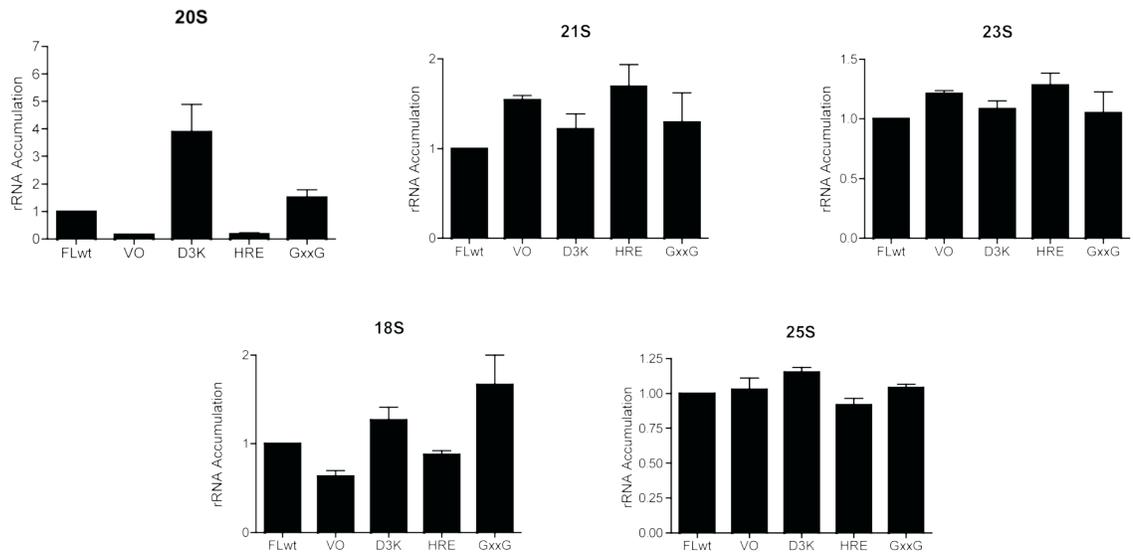


Figure 23. Mutations in Dim2 Disrupt Nob1-Dependent D-Site Cleavage. A) Northern blotting (time points taken at 0 hr, 8 hr, and 12 hr) shows an accumulation of 20S rRNA with the D3/K mutation in Dim2 and decreases with a Dim2 deletion and the HR/E mutation. GxxG gives an intermediate phenotype. B) Quantitation of the Northern Blots at 8h.

Nob1 depletion only affects cytoplasmic D-site cleavage, we do not think that the Dim2 requirement for A₂ cleavage involves the interaction with Nob1.

Dim2 recruits Nob1 to pre-ribosomes and properly aligns Nob1 for D-site cleavage.

Previous research has shown that Dim2 co-purifies with both early and late ribosome assembly factors, indicative of Dim2 association with early and late pre-ribosomal particles (Gavin *et al.*, 2002; Grandi *et al.*, 2002; Schafer *et al.*, 2006; Vanrobays *et al.*, 2004; Vanrobays *et al.*, 2008). Contrastingly, Nob1 mostly associates with late ribosome assembly factors (Schafer *et al.*, 2003; Schafer *et al.*, 2006), indicating that Dim2 binds earlier than Nob1 to pre-ribosomes. Because Dim2 appears to bind early in ribosome biogenesis, we wanted to test whether Dim2 was required to recruit Nob1 to pre-ribosomes.

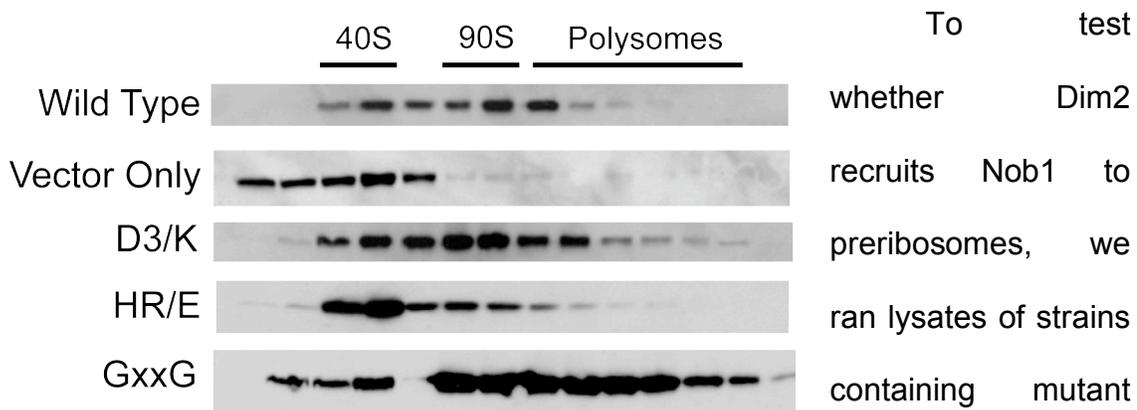


Figure 24. Dim2 mutations change Nob1 localization on pre-ribosomes. Nob1-TAP; Dim2::Gal strains grown in glucose for 8hrs were fractionated on sucrose gradients. Nob1 localizations on pre-ribosomes were detected through western blots. Deletion of Dim2 significantly decreases Nob1 binding to pre-ribosomal particles; while, Dim2 mutations cause various phenotypes. D3/K and GxxG lead to an increase in the amount of Nob1 bound to 90S particles. HR/E leads to a decrease in Nob1 bound to 90S particles and an increase in Nob1 bound to pre-40S subunits that cannot be incorporated into mature ribosomes.

Dim2 (endogenous Dim2 was depleted for 8h in glucose) over 10-50% sucrose gradients

and detected Nob1 localization via Western blots. As seen previously (Soudet *et al.*, 2010; Tone & Toh-E, 2002), in the presence of wild type Dim2, Nob1 is found

on 43S pre-ribosomes, 90S pre-ribosomes, and polysomes (Figure 24). When cells are depleted of Dim2, Nob1 is found in the lightest fractions of the sucrose gradient indicating Nob1 is no longer associated with larger ribosomal particles in the absence of Dim2. There is still remaining association with a 40S-like particle; however, since no 20S rRNA is produced in the absence of Dim2 (Figure 23), we hypothesize that this particle contains a 21S rRNA, which does not mature to a functional 20S rRNA or no RNA at all. Importantly, the increase in unbound Nob1 in the absence of Dim2 shows that Dim2 is involved in recruiting Nob1 to preribosomes. However, since Nob1 is still partially associated with a 40S-like ribosomal particle, Nob1 can bind preribosomes independent of Dim2 through other protein-protein interactions or through its own RNA binding activity.

Also shown in Figure 24, the mutations in the middle KH domain of Dim2 have various effects on Nob1 localization on pre-ribosomes. All mutations result in a small increase in the amount of free Nob1. The HR/E mutant shows an increase in the amount of Nob1 bound to a 40S-like particle with a decrease in the amount of Nob1 associated with 90S and a complete abolishment of Nob1 associated with polysomes. These data are not surprising considering the northern blot showed that this mutation results in a complete loss of 20S rRNA. Taken together these data suggest that this 40S-like particle contains either a 21S rRNA or 23S rRNA, as seen produced in the northern blots (figure 23) that cannot be incorporated in functional ribosomes. As expected, the D3/K and GxxG mutants accumulate Nob1 in polysomes, as previously seen for other mutants, where 20S rRNA is accumulated (Soudet *et al.*, 2010).

Taken together, these data show that Dim2 is involved in Nob1 recruitment to pre-ribosomes. Additionally, Nob1 is present in pre-ribosomes in the presence of the D3/K and GxxG mutations in Dim2, suggesting that the observed 20S accumulation reflects a poorly functional Nob1 when Dim2 is mutated to no longer bind Nob1. Without the appropriate interaction between Dim2 and Nob1, D-site cleavage cannot occur properly and Nob1 remains bound to the small subunit of polyribosomes.

Discussion

Dim2•Nob1 interaction is essential for ribosome assembly.

In the previous chapter, we showed a direct interaction between Dim2 and Nob1. Truncation analysis implicated the central divergent KH domain, and mutation analysis directly implicated the tryptophan in the interaction loop as a residue directly involved in this interaction. Here we show by mutational analysis that several highly conserved, surface-exposed charged residues also disrupt Nob1 binding *in vitro*. Subsequent *in vivo* analysis determined that several of these conserved residues are essential for Dim2 function in ribosome assembly; however, further analysis revealed that these mutations disrupt different Dim2 functions in ribosome assembly.

Dim2 roles in ribosome assembly.

(a) *Dim2 recruits Nob1 to pre-ribosomes and is required for cleavage at site D*

Density gradient centrifugation shows that depletion of Dim2 leads to the majority of Nob1 no longer bound to pre-ribosomes. Northern blot analysis of the D3/K and GxxG Dim2 mutants shows decreased D-site cleavage, although density gradient centrifugation indicates that Nob1 remains bound to 43S-pre-ribosomes. Taken together, this data suggest that a) Dim2 helps recruit Nob1 to pre-ribosomes and b) that Dim2 must additionally affect Nob1 cleavage activity in a more subtle way.

The requirement for the Dim2•Nob1 interaction for proper D-site cleavage could be due to a number of reasons. First, Dim2 could directly regulate Nob1 cleavage activity, resulting in a loss of cleavage when the interaction is disrupted. We set out to test this model in a simple system using recombinant Nob1 and *in vitro* transcribed RNA (Lamanna & Karbstein, 2011). However, in this system Dim2 has no additional effect on Nob1 cleavage activity (AC. Lamanna, personal communication, data not shown), suggesting that this model is less likely.

On the other hand, the requirement of this protein-protein interaction for proper D-site cleavage could be indirect, through separate assembly factors. *In vitro* pull-down interaction assay analysis of all assembly factors that bind to late 43S pre-ribosomes (Tsr1, Rio2, Enp1, Ltv1, Dim1 and Nob1; Chen *et al.*, 2003; Connolly *et al.*, 2008; Fassio *et al.*, 2010; Fatica *et al.*, 2004; Gelperin *et al.*, 2001; Granneman *et al.*, 2010; Schafer *et al.*, 2003; Vanrobays *et al.*, 2003) shows that Dim2 only binds directly to Nob1 and Rio2 (M.G. Campbell & K. Karbstein, 2010). This suggests that Rio2 is a candidate protein that mediates regulation of this protein-protein interaction. Additionally, indirect mediation of this

interaction could involve ribosomal proteins or other assembly factors not stably bound to pre-ribosomes, such as Fap7 or Rio1 (Granneman *et al.*, 2005; Strunk & Karbstein, 2009; Vanrobays *et al.*, 2003). However, additional experiments are needed to distinguish between these possibilities.

(b) Dim2 is required for cleavage at site A₂

Northern analysis of the HR/E mutant shows a severe decrease in 20S production, suggesting that this mutation disrupts A₂ cleavage. This suggests that the diverged KH domain of Dim2 interacts with additional assembly factors that affect early processing steps. Using the same *in vitro* binding assay described here for Dim2•Nob1 binding, we tested for, but could not determine, an interaction with Rcl1, the putative A₂ site nuclease (data not shown).

The idea that mutations in different regions of the central diverged KH domain of Dim2 disrupt different steps in ribosome assembly suggests that the protein-interaction surface is highly dynamic and is remodeled during ribosome assembly. This could also be due to Dim2 acting as a dimer (Chapter II), but further analysis would have to be done to determine if Dim2 can bind multiple assembly factors at once.

(c) Dim2 is required for 40S export and SSU processome assembly

Previously, it was demonstrated that the presence of a nuclear export sequence (NES) in the C-terminal KH domain is required to efficiently export the 40S subunit for cytoplasmic maturation (Vanrobays *et al.*, 2008). Additionally, SSU processome assembly is interrupted after Dim2 depletion (Vanrobays *et al.*, 2004), suggesting additional roles in assembly for Dim2.

(d) A role for eukaryotic Dim2 in translation initiation?

The structure of a complex of archeal Dim2 (aDim2) bound to aIF2 α suggests a role for aDim2 in translation initiation (Jia *et al.*, 2010). However, there are major differences between the archeal Dim2 and eukaryotic Dim2. First, aDim2 lacks the N-terminal KH domain of eukaryotic Dim2, which we have shown is required for dimerization. While this KH domain is not essential for growth, deletion of this domain is detrimental (Chapter II). Second, aDim2 has a conserved KH domain in place of the divergent KH domain found in eukaryotic Dim2. In the solved structure, this conserved KH domain is bound to the anti-Shine-Dalgarno sequence (not conserved in eukaryotes). However, we have shown here that the divergent KH domain in eukaryotic Dim2 interacts with the endonuclease Nob1 (and possibly with multiple other assembly factors). While we cannot rule out a role in translation initiation, these noticeable differences between aDim2 and eukaryotic Dim2 are suggestive of slightly different roles. Dim2 might have a role in translation initiation; however, additional experiments are required to conclude the role of Dim2 in translation initiation.

Materials and Methods

Yeast Strains and yeast expression plasmids: Yeast strain YKK192, created from the Open Biosystems Nob1-TAP strain, has a galactose-inducible promoter inserted in front of the Dim2 open reading frame. It was generated using PCR-based recombination (Longtine *et al.*, 1998), and correct insertion was verified by PCR and Western analysis. The Dim2 open reading frame, including an N-

terminal HA-tag, was cloned into pRS415cyc1 and pRS416TEF using oligonucleotides listed in Table 2.

Protein expression plasmids: Dim2 open reading frame, N-terminal, and C-terminal deletion constructs were cloned into pSV272 for protein expression in Rosetta competent cells using oligonucleotides listed in Table 2.

Mutagenesis: Dim2 mutants were generated using the Quikchange protocol. See chart below for mutagenesis primers used in cloning.

Expression and Purification of Dim2: His-MBP-tagged Dim2 plasmid DNA (pSV272; wild type, mutants, and truncations) was freshly transformed into Rosetta cells. Protein expression was carried out as previously described (Lamanna & Karbstein, 2009). Cells were lysed in 50 mM NaPO₄ (pH 8.0), 150 mM NaCl, and 0.5 mM PMSF and purified via Ni-NTA chromatography using 1ml HisTrap Columns (GE Healthcare). Ni eluate fractions containing Dim2 were pooled and dialyzed 3 h (or overnight) into 50 mM Tris (pH 8.0), 150 mM NaCl, 1mM DTT (Buffer A) at 4°C. Dialyzed protein was loaded onto a MonoS column equilibrated in Buffer A and eluted in a salt gradient to a final concentration of 40% of Buffer B (50 mM Tris (pH 8.0), 2 M NaCl, 1 mM DTT) over 12 column volumes. Fractions containing Dim2 were pooled and concentrated and further purified over a Superdex200 column in 50mM HEPES (pH 8.0), 200 mM NaCl, 1mM DTT. Dim2-containing fractions were pooled and concentrated and flash frozen and stored at -80°C. Protein concentration was determined with a nanodrop spectrophotometer using Beer's law.

Dim2 mutant proteins were purified using the same protocol as the wild type protein with the exception that the mutant proteins bound to the MonoQ ion-exchange column.

Transcriptions: rDNA fragments were cloned into pUC19 between XmaI and PstI sites as described (Lamanna & Karbstein, 2009). 50 mL overnight cultures were grown in LB medium with Ampicillin and plasmid DNA isolated using a maxiprep kit (Qiagen). Plasmid DNA was digested with BtsI (NEB), phenol: chloroform extracted and resuspended in 200 μ L of water. Transcriptions were carried out as described (Lamanna & Karbstein, 2009a) using the entire digested rDNA template in a final volume of 5 μ L. The reaction was terminated by adding 0.5 μ L of 0.5 M EDTA (pH 8.0), and RNA was precipitated overnight at -20°C by adding 0.55 μ L of 3 M sodium acetate and 16.5 μ L ethanol. RNA was purified using an RNA Maxi Kit (Qiagen). To obtain body-labeled transcripts reactions were carried out as previously described (Lamanna & Karbstein, 2009).

RNA Binding Assay: RNA was folded as described (Lamanna & Karbstein, 2009). Dim2 was pre-incubated at 30°C for 15-20 minutes before being RNA was added to binding tubes. 10 μ l binding reactions were incubated at 30°C for 2 hours and then immediately loaded onto an 8% Acrylamide/THEM (pH 8.0) gels (33 mM Tris, 67 mM HEPES, 1 mM EDTA, 10 mM MgCl₂) after addition of 2 μ l of RNA loading dye (0.1% xylene cyanol, 0.1% bromophenol blue, 45% glycerol). Gels were run at 250V for ~2 hours, dried and exposed to a phosphoscreen overnight. Signal was detected using Typhoon scanner and quantification was performed using ImageQuant software.

In vitro Pull Down Assays: 10 μ M recombinant MBP-tagged Dim2 (or MBP-tagged Nob1) was incubated with 10 μ M or 40 μ M untagged Nob1 (or untagged Dim2) for 30 minutes at room temperature (in size-exclusion buffer from purification). Pre-equilibrated amylose resin was added to protein mixture and incubated on a shaker for 30 minutes at room temperature. The protein-amylose resin mixture was applied to a disposable column and the flow through was collected by centrifugation for a couple of seconds. The column was washed with 1mL of buffer and centrifuged for a couple of seconds to collect all residual buffer. Bound proteins were eluted with a 50mM maltose solution (50 mM maltose in size-exclusion buffer) and analyzed via SDS-PAGE.

Dim2 Modeling: The open reading frame sequence for *S. cerevisiae* Dim2 (YOR145C; Chromosome XV: 606172 to 605348) was submitted to two protein structure homology modeling servers, SwissModel (<http://swissmodel.expasy.org/>) (Arnold, *et al.*, 2006; Bordoli *et al.*, 2009; Kiefer, *et al.*, 2009) and 3Djigsaw (<http://bmm.cancerresearchuk.org/~3djigsaw/>) (Bates, *et al.*, 2001). Both servers modelled the c-terminal two-thirds of the sequence to the crystal structure of Dim2p from *Pyrococcus horikoshii* OT3 (PDB entrant 2E3U), an archaeal homolog of *S. cerevisiae* Dim2 (Jia, *et al.*, 2007). The models were analyzed using Pymol software.

Northern Analysis: YKK192 was transformed with Dim2 constructs in pRS416TEF (or empty plasmid vector). Transformants were grown in galactose

in mid log phase growth for at least 12 hours before being grown in YPD for sample collection. 10 OD units of cells were collected 0, 8 and 12 hours after switch to glucose media. Cells were pelleted and washed twice in water before being stored at -80 °C. For RNA extraction, cells were resuspended in 400 µl TES buffer (10 mM Tris (pH 7.5), 10 mM EDTA, 0.5% (w/v) SDS). 400 µl of acid phenol was added and samples were incubated at 65 °C for 45 minutes. The acid phenol extraction was repeated, followed by a phenol/chloroform extraction. RNA was ethanol precipitated at -80 °C for an hour and resuspended in nuclease-free water and stored at -20 °C. RNAs were separated on a 1 % agarose/formaldehyde gel, transferred to Amersham Hybond-N membrane (GE Healthcare) by passive transfer, and crosslinked at high efficiency using optimal conditions of the UV-crosslinker (FB-UV XL-1000; Fisher Scientific). To detect transferred RNAs 10 µM Northern probe (IDT, Table 2) was end-labeled using T4 polynucleotide kinase (NEB) and γ -P³²-ATP. The 5 µl reaction was incubated at 37 °C for 1 hour, diluted with 25 µl water, and purified using G-50 columns (GE Healthcare). Membranes were washed twice with boiling 0.1% SDS for 10 minutes at 65 °C followed by a 1-4 hour incubation at 37 °C in prehybridization buffer (7.5X Denhardt's Solution, 5X SSPE, 0.75 M NaCl, 75 mM NaCitrate, pH 7.0, 0.1% SDS, single stranded DNA). Then, 5 µl of the probe (in 500 µl prehybridization buffer) was denatured at 95 °C for 3 minutes and added to the membrane. Membranes were incubated with probes at 37 °C overnight. Membranes were washed in 5X SSPE, 0.1% SDS for 10 minutes at 37 °C followed by a wash in 0.5X SSPE, 0.1% SDS for 10 minutes at 37 °C.

Membranes were exposed to a phospho-screen or film for 2-6 hours (depending on the probe used), and analyzed using ImageQuant software.

Sucrose Gradients: YKK192 was transformed with Dim2 constructs in pRS416TEF (or empty plasmid vector). Overnight cultures were shifted to YPD for 8 hours prior to sample collection. After 7.5 hours, 100OD units of cells were incubated with 100 µg/µl cycloheximide at 30 °C while shaking and subsequently pelleted and resuspended in gradient lysis buffer containing 100 µg/µl cycloheximide (20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl₂) before being flash frozen. Cells were lysed under liquid nitrogen by grinding with mortar and pestle before being thawed and loaded onto 10-50% 5 ml sucrose gradients containing 100 µg/µl cycloheximide. Gradients were made by layering five sucrose-containing solutions with each layer being frozen at -80 °C for 15 minutes before the next one was added. Frozen gradients were thawed at 4 °C for 1 hour prior to sample application, to allow for thawing and smoothing of the gradient. Gradients were centrifuged at 40,000 rpm for 2 hours. 375 µl fractions were collected and half of the volume was TCA precipitated analyzed by western blots using IgG antibody to detect the TAP tag on Nob1. RNA was precipitated from the remaining volume of the fractions using isopropanol and then analyzed via northern blotting (described above).

Table 2: Primers Used for Yeast Work

Primers for Cloning into pSV272	
FLwt 5' (Sfo1)	GATCGAGGCGCCATGGTTGCGCCTA
FLwt 3' (HindIII)	TCAGACAAGCTT TTAGTAGCGTTCT TTAATCTAG
N-terminal Deletion 5' (Sfo1)	GTAGTAGGCGCCACACAGGGTAACA AAATAAAATTTGAATCGAG
C-terminal Deletion 3' (HindIII)	CAAGTAAAGCTTTCAATCTAGAATCA ATAGTGAGATAGAATCG

Primers for Cloning into pRS415cyc1/416pTEF	
Spe1HADim2FL 5'	GCCGCCACTAGTATGTATCCTTATGA CGTGCCTGACTATGCCAGCCTGGGA GGACCTATGGTTGCGCCTACTGCTTTG
Same 3' Primer Listed for pSV272	

Primers for Creating Galactose-Inducible Strain	
KAN-Gal Cassette 5'	CCTCTTACGGAAAGTGCGAGCGGGG AATTCGAGCTCGTTTAAAC
KAN-Gal Cassette 3'	GCCTTTTTCAAAGCAGTAGGCGCAA CCATCATTTTGAGATCCGGGTTTT

Mutagenesis Primers	
Dim2 D3/K	CTTTACCTTAGGGTTTAAACTCAAG AAGTCTATCGCACTATTGAG
Dim2 HR/E	GAAAGATCAGGTTTCCACCAGAGGA AATGACACCCTTGAGGAAC
Dim2 GXXG	GAATGACACCCTTGAGGGGCAAGGA CGGAAAAATTTATCCTCCTTTGG
Dim2 WK/A	CACCCTTGAGGAACAGTGCTAC GGCAATTTATCCTCCTTTGG

Northern Probes	Sequence (5'-3')
Probe B (20S)	GCT CTC ATG CTC TTG CC
Probe Y (25S)	GGGCAGGCTGCAGCTTCTACCAG
Probe 18S	CAT GGC TTA ATC TTT GAG AC
Probe C (between A2-A3)	ATG AAA ACT CCA CAG TG
Probe U2	CAGATACTACACTTG
Probe Z (3' end of 5' ETS)	GGA AAC AGC TGA AAT TCC

References

- Arnold, K., Bordoli, L., Kopp, J., & Schwede, T. (2006). The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics (Oxford, England)*, *22*(2), 195-201.
doi:10.1093/bioinformatics/bti770
- Bates, P. A., Kelley, L. A., MacCallum, R. M., & Sternberg, M. J. (2001). Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. *Proteins, Suppl 5*, 39-46.
- Bernstein, K. A., Gallagher, J. E., Mitchell, B. M., Granneman, S., & Baserga, S. J. (2004). The small-subunit processome is a ribosome assembly intermediate. *Eukaryotic Cell*, *3*(6), 1619-1626. doi:10.1128/EC.3.6.1619-1626.2004
- Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J., & Schwede, T. (2009). Protein structure homology modeling using SWISS-MODEL workspace. *Nature Protocols*, *4*(1), 1-13. doi:10.1038/nprot.2008.197
- Chen, W., Bucaria, J., Band, D. A., Sutton, A., & Sternglanz, R. (2003). Enp1, a yeast protein associated with U3 and U14 snoRNAs, is required for pre-rRNA processing and 40S subunit synthesis. *Nucleic Acids Research*, *31*(2), 690-699.
- Connolly, K., Rife, J. P., & Culver, G. (2008). Mechanistic insight into the ribosome biogenesis functions of the ancient protein KsgA. *Molecular Microbiology*, *70*(5), 1062-1075. doi:10.1111/j.1365-2958.2008.06485.x

- Fassio, C. A., Schofield, B. J., Seiser, R. M., Johnson, A. W., & Lycan, D. E. (2010). Dominant mutations in the late 40S biogenesis factor Ltv1 affect cytoplasmic maturation of the small ribosomal subunit in *Saccharomyces cerevisiae*. *Genetics*, *185*(1), 199-209. doi:10.1534/genetics.110.115584
- Fatica, A., Tollervey, D., & Dlakic, M. (2004). PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. *RNA (New York, N.Y.)*, *10*(11), 1698-1701. doi:10.1261/rna.7123504
- Fromont-Racine, M., Senger, B., Saveanu, C., & Fasiolo, F. (2003). Ribosome assembly in eukaryotes. *Gene*, *313*, 17-42.
- Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Merino, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edlmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., & Superti-Furga, G. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature*, *415*(6868), 141-147. doi:10.1038/415141a
- Gelperin, D., Horton, L., Beckman, J., Hensold, J., & Lemmon, S. K. (2001). Bms1p, a novel GTP-binding protein, and the related Tsr1p are required for distinct steps of 40S ribosome biogenesis in yeast. *RNA (New York, N.Y.)*, *7*(9), 1268-1283.

- Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., Schafer, T., Kuster, B., Tschochner, H., Tollervey, D., Gavin, A. C., & Hurt, E. (2002). 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. *Molecular Cell*, *10*(1), 105-115.
- Granneman, S., Nandineni, M. R., & Baserga, S. J. (2005). The putative NTPase Fap7 mediates cytoplasmic 20S pre-rRNA processing through a direct interaction with Rps14. *Molecular and Cellular Biology*, *25*(23), 10352-10364. doi:10.1128/MCB.25.23.10352-10364.2005
- Granneman, S., Petfalski, E., Swiatkowska, A., & Tollervey, D. (2010). Cracking pre-40S ribosomal subunit structure by systematic analyses of RNA-protein cross-linking. *The EMBO Journal*, *29*(12), 2026-2036. doi:10.1038/emboj.2010.86
- Jia, M. Z., Horita, S., Nagata, K., & Tanokura, M. (2010). An archaeal Dim2-like protein, aDim2p, forms a ternary complex with a/eIF2 alpha and the 3' end fragment of 16S rRNA. *Journal of Molecular Biology*, *398*(5), 774-785. doi:10.1016/j.jmb.2010.03.055
- Jia, M. Z., Ohtsuka, J., Lee, W. C., Nagata, K., & Tanokura, M. (2007). Crystal structure of Dim2p: A preribosomal RNA processing factor, from pyrococcus horikoshii OT3 at 2.30 Å. *Proteins*, *69*(2), 428-432. doi:10.1002/prot.21381
- Kiefer, F., Arnold, K., Kunzli, M., Bordoli, L., & Schwede, T. (2009). The SWISS-MODEL repository and associated resources. *Nucleic Acids Research*, *37*(Database issue), D387-92. doi:10.1093/nar/gkn750

- Kos, M., & Tollervey, D. (2010). Yeast pre-rRNA processing and modification occur cotranscriptionally. *Molecular Cell*, 37(6), 809-820.
doi:10.1016/j.molcel.2010.02.024
- Lamanna, A. C., & Karbstein, K. (2009a). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings of the National Academy of Sciences of the United States of America*, 106(34), 14259-14264. doi:10.1073/pnas.0905403106
- Lamanna, A. C., & Karbstein, K. (2009b). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings of the National Academy of Sciences of the United States of America*, 106(34), 14259-14264. doi:10.1073/pnas.0905403106
- Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., & Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *saccharomyces cerevisiae*. *Yeast (Chichester, England)*, 14(10), 953-961.
doi:2-U
- Osheim, Y. N., French, S. L., Keck, K. M., Champion, E. A., Spasov, K., Dragon, F., Baserga, S. J., & Beyer, A. L. (2004). Pre-18S ribosomal RNA is structurally compacted into the SSU processome prior to being cleaved from nascent transcripts in *saccharomyces cerevisiae*. *Molecular Cell*, 16(6), 943-954. doi:10.1016/j.molcel.2004.11.031

- Schafer, T., Maco, B., Petfalski, E., Tollervey, D., Bottcher, B., Aebi, U., & Hurt, E. (2006). Hrr25-dependent phosphorylation state regulates organization of the pre-40S subunit. *Nature*, *441*(7093), 651-655. doi:10.1038/nature04840
- Schafer, T., Strauss, D., Petfalski, E., Tollervey, D., & Hurt, E. (2003). The path from nucleolar 90S to cytoplasmic 40S pre-ribosomes. *The EMBO Journal*, *22*(6), 1370-1380. doi:10.1093/emboj/cdg121
- Senapin, S., Clark-Walker, G. D., Chen, X. J., Seraphin, B., & Daugeron, M. C. (2003). RRP20, a component of the 90S preribosome, is required for pre-18S rRNA processing in *saccharomyces cerevisiae*. *Nucleic Acids Research*, *31*(10), 2524-2533.
- Soudet, J., Gelugne, J. P., Belhabich-Baumas, K., Caizergues-Ferrer, M., & Mougin, A. (2010). Immature small ribosomal subunits can engage in translation initiation in *saccharomyces cerevisiae*. *The EMBO Journal*, *29*(1), 80-92. doi:10.1038/emboj.2009.307
- Strunk, B. S., & Karbstein, K. (2009). Powering through ribosome assembly. *RNA (New York, N.Y.)*, *15*(12), 2083-2104. doi:10.1261/rna.1792109
- Thomas, F., & Kutay, U. (2003). Biogenesis and nuclear export of ribosomal subunits in higher eukaryotes depend on the CRM1 export pathway. *Journal of Cell Science*, *116*(Pt 12), 2409-2419. doi:10.1242/jcs.00464
- Tone, Y., & Toh-E, A. (2002). Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *saccharomyces cerevisiae*. *Genes & Development*, *16*(24), 3142-3157. doi:10.1101/gad.1025602

- Tschochner, H., & Hurt, E. (2003). Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends in Cell Biology*, 13(5), 255-263.
- Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., & Lafontaine, D. L. (2004). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. *RNA (New York, N.Y.)*, 10(4), 645-656.
- Vanrobays, E., Gelugne, J. P., Gleizes, P. E., & Caizergues-Ferrer, M. (2003). Late cytoplasmic maturation of the small ribosomal subunit requires RIO proteins in *saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 23(6), 2083-2095.
- Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., & Lafontaine, D. L. (2008a). TOR regulates the subcellular distribution of DIM2, a KH domain protein required for cotranscriptional ribosome assembly and pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.
doi:10.1261/rna.1176708
- Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., & Lafontaine, D. L. (2008b). TOR regulates the subcellular distribution of DIM2, a KH domain protein required for cotranscriptional ribosome assembly and pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.
doi:10.1261/rna.1176708
- Venema, J., & Tollervey, D. (1999). Ribosome synthesis in *saccharomyces cerevisiae*. *Annual Review of Genetics*, 33, 261-311.
doi:10.1146/annurev.genet.33.1.261

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences*, 24(11), 437-440.

Warner, J. R., Vilardell, J., & Sohn, J. H. (2001). Economics of ribosome biosynthesis. *Cold Spring Harbor Symposia on Quantitative Biology*, 66, 567-574.

CHAPTER IV

Dim2 rRNA Binding Site

Introduction

Of the essential assembly factors involved in ribosome assembly, more than 40 proteins are RNA binding proteins with undetermined functions. In an attempt to understand the function of these proteins, research has begun to locate binding sites on pre-ribosomes. This idea propelled research from the Tollervey lab that crosslinked proteins to rRNA, and the binding sites of many RNA binding proteins have been mapped in that way (Bohnsack *et al.*, 2009; Granneman *et al.*, 2009; Granneman *et al.*, 2010). However, no cross-links were observed between Dim2 and rRNA, and thus it is the only protein involved in late-cytoplasmic maturation of the pre-40S subunit with an unknown RNA binding site. Furthermore, Dim2 is unique as it associates with pre-ribosomes early in the nucleolus and remains associated through export into the cytoplasm for late maturation steps (Vanrobays *et al.*, 2004; Vanrobays *et al.*, 2008). Thus, characterizing its binding site might reveal information about changes in pre-rRNA during assembly, as well as constants.

One such pre-rRNA conformational change has been determined to be involved in Nob1 regulation. Nob1 is the endonuclease responsible for late cytoplasmic D-site cleavage of pre-rRNA, resulting in the mature 3' end of the 18S rRNA in the small subunit (Fatica *et al.*, 2004; Lamanna & Karbstein, 2009).

Although Nob1-dependent D-site cleavage occurs in the cytoplasm, Nob1 is associated with early, nucleolar pre-40S particles. Since this early association with pre-ribosomes does not immediately result in nucleolar D-site cleavage, Nob1 activity must be spatially or temporally regulated. Our lab has recently shown that prior to A₂ cleavage, nucleotides in ITS1 base pair with a region of helix 44, forming an alternative structure that is not conducive to D-site cleavage

(Figure 25).

Following A₂ cleavage and subsequent removal of the ITS1 sequence, the mature helix 44 is formed.

Additionally, DMS

probing has shown that Nob1 binds to this pre-A₂ alternative rRNA conformation differently than the post-A₂ rRNA conformation, shown in Figure 25, suggesting this rRNA conformational switch as a regulation checkpoint in Nob1 activity. However, understanding how this conformational change takes place and exactly how this change in rRNA conformation affects Nob1 positioning on pre-ribosomes requires further research to completely understand regulation of Nob1 cleavage activity. In the previous chapters we set out to determine if Dim2 interacts with Nob1 and how Dim2 could be involved in Nob1 regulation. We

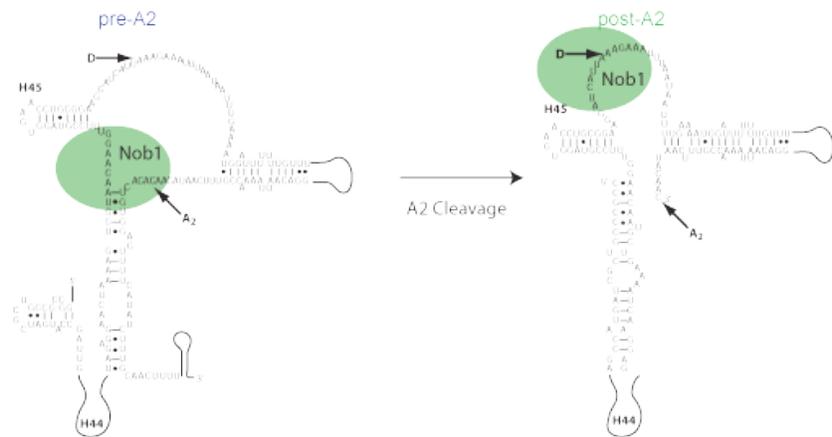


Figure 25: Nob1 binds rRNA conformations differently. rRNA adopts different conformations before and after A₂ cleavage. DMS probing has shown that Nob1 binds differently to these two rRNA conformations.

found that Dim2 is partially involved in recruitment of Nob1 to pre-ribosomes as well as in proper positioning of Nob1 on pre-ribosomes around D-site cleavage (Chapter III). Since Dim2 interacts with Nob1, and Nob1 binds rRNA around cleavage site D, Dim2 is expected to bind in the vicinity of cleavage site D. In this chapter we wanted to further characterize where Dim2 binds on pre-ribosomal rRNA, especially relative to the Nob1 rRNA binding site. Additionally we wanted to see if or how this interaction with rRNA might change during ribosome assembly.

Results

In previous chapters we have shown a direct interaction between Dim2 and Nob1 mediated by the central KH domain in Dim2, specifically involving residues D167/169/170 as well as the substrate binding loop of KH domains, consisting of residues 111-114 in Dim2. Mutations, in which these three aspartic acids were changed to lysines, or which replace the noncanonical tryptophan-containing loop with a classical GxxG loop, abolish binding to Nob1 *in vitro* and disrupts efficient Nob1-dependent D-site cleavage *in vivo*, resulting in an accumulation of a 20S rRNA intermediate. Additionally, Nob1 localization on pre-ribosomes was affected by these Dim2 mutations resulting in an accumulation of Nob1 on pre-ribosomes. Considering that Dim2 affects Nob1 localization on pre-ribosomes as well as D-site cleavage activity, we wanted to test whether or not Dim2 directly affected how Nob1 binds to rRNA. Furthermore, we wanted to determine where Dim2 binds pre-rRNA.

Dim2 Specifically Binds to Helix 44 in Pre-rRNA Constructs

To determine the Dim2 rRNA preference, gel-shift assays were carried out with the rRNA constructs shown in Figure 26A. The rRNA constructs either begin at helix 44 or helix 45 and continue to cleavage site A₂ (212 nucleotides 3' to cleavage site D) or 278 nucleotides past cleavage site D (~cleavage site A₃). To

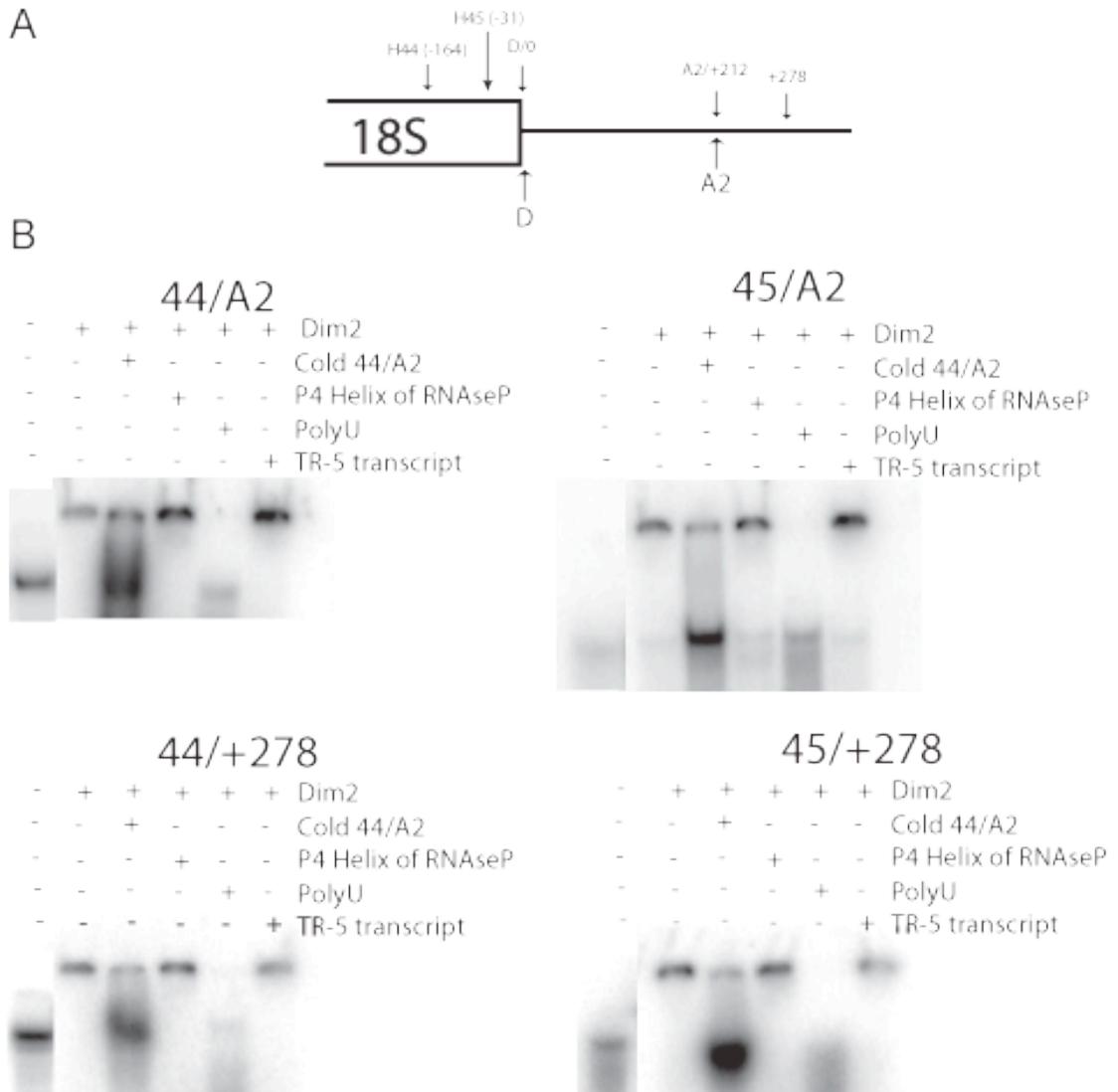


Figure 26: Dim2 specifically binds rRNA. A) Constructs of rRNA were cloned beginning at helix 44 or 45 of the 3' end of the 18S rRNA and ending at A₂ cleavage site (+212 bases downstream of D-site cleavage) or +278 bases downstream of D-site cleavage. B) Gel-shift mobility assays show that Dim2 binding to rRNA constructs is specific.

determine if Dim2 binding to these rRNA constructs was specific, gel-shift assays were carried out as described in Materials and Methods.

As can be seen in Figure 26b, Dim2 binds the pre-rRNA fragments, as the RNA is shifted to a lower electrophoretic mobility (compare first and second lanes in each panel). To test if these interactions are specific, unlabeled RNAs were used as competitors. Because H44/A₂ rRNA has the highest affinity for Dim2 it was used as a positive control. In addition, we used polyU RNA, the P4 helix of RNaseP (a stem-loop ~25 bases long) and a run-on transcript (TR-5) obtained when the RNaseP-containing plasmid was not linearized prior to transcription. As shown in Figure 26, unlabeled H44/A₂ was able to compete off most of Dim2 bound to the labeled rRNA constructs. As expected, the P4 helix of RNaseP and the TR-5 transcript did not compete with Dim2 binding at all, showing that Dim2 binding is specific to rRNA sequences. Interestingly, polyU also interfered with Dim2 binding to these rRNA constructs. Importantly, the sequence of ITS1 surrounding the D cleavage-site is rich in uracils, indicating perhaps that Dim2 binds near these sequences. Nevertheless, these data show that Dim2 specifically binds to these rRNA sequences containing cleavage site D.

Dim2 Binds H44 and ITS1 Around Cleavage Site D.

To learn more about where in H44 and ITS1 Dim2 bound to rRNA, we compared the affinity of a series of rRNA fragments to Dim2. These rRNA affinities are shown in Table 3. Comparing the affinities for the three RNAs with 3'-ends at site A₂ (the 3'-end of the Nob1-Dim2 substrate 20S rRNA) reveals that

addition of H44 strengthens binding 4-fold ($K_{1/2}$ values of .24 and .86 μM for H44/ A_2 and H45/ A_2 rRNAs, respectively). This indicates that interactions are made

	$K_{1/2}$ (μM)	St. Error (μM)
H44/A_2	0.24	0.05
H44/+278	0.64	0.14
H45/A_2	0.86	0.72
H45/+278	0.74	0.25
D/A_2	1.4	0.43

Table 3: Dim2 affinities for rRNA constructs as determined by gel-shift assays.

with regions within H44. Furthermore, interactions are also made with residues between H45 and site D, as the D/ A_2 RNA binds 2-fold weaker still than the H45/ A_2 RNA ($K_{1/2}$ values of .86 and 1.4 μM for H45/ A_2 and D/ A_2 rRNAs, respectively). Adding sequence in ITS1 3'-to site A_2 does not help binding, indicative that Dim2 does not make interactions with this region of the RNA ($K_{1/2}$ values of .86 and .74 μM for H45/ A_2 and H45/+278 rRNAs, respectively).

We have previously shown that the H44/+278 rRNA adopts a different structure than the other rRNAs shown in this Table (Figure 25). This alternative structure represents a complex formed early in ribosome assembly, when Dim2 (and Nob1) are already bound to pre-ribosomal particles, and is stabilized by base-pairing interactions between nucleotides 217-237. We have previously shown that the role of this earlier structure is to prevent premature Nob1-dependent cleavage at site D, and to order the cleavages at sites A_2 and D. Comparing the affinity for H44/ A_2 (in the later rRNA structure) to the affinity for H44/+278 (in the earlier structure) reveals that Dim2 has a preference for the later rRNA structure, akin to, but more pronounced than Nob1 ($K_{1/2}$ values of .24 and .64 μM for H44/ A_2 and H44/+278 rRNAs, respectively). The identical

affinities for H45/A₂ and H45/+278 rRNAs (which are both in the later rRNA structure) indicate that the 3-fold stronger binding of H44/A₂ rRNA is due to the preference for the earlier structure.

DMS probing reveals a complex mode of Dim2 interactions with rRNA

To confirm and extend the quantitative analysis of Dim2 RNA binding, we used *in vitro* DMS structure probing of rRNA in the presence and absence of Dim2. DMS methylates unpaired adenosines and cytosines, which will subsequently result in elongation termination of the primer extension reaction. Addition of protein can protect sites of direct interaction, and it can also lead to changes in the RNA structure. These changes in the rRNA structure can result in both enhancements as well as protections. As shown in Figure 27a in the presence of Dim2, DMS methylation decreased on two bases resulting in a decrease in the primer extension signal at these two locations. These two bases are located in H44 and just 5'- to site D, respectively. While we cannot exclude the possibility that these protections result from changes in the rRNA structure, the protection near site D is found on a residue that is directly contacted by archeal Dim2 in a recent crystal structure (Jia *et al.*, 2007). The second residue was not part of the crystal structure.

In addition to these protections, several sites of enhancements were found (Figure 27a). These cluster in two regions, one in H44, and one near site D, and their location is mapped on the secondary and tertiary structure of this rRNA element, as found in the recent crystal structure in Figure 27b and c. These sites of enhancement indicate that at these locations the rRNA structure changes

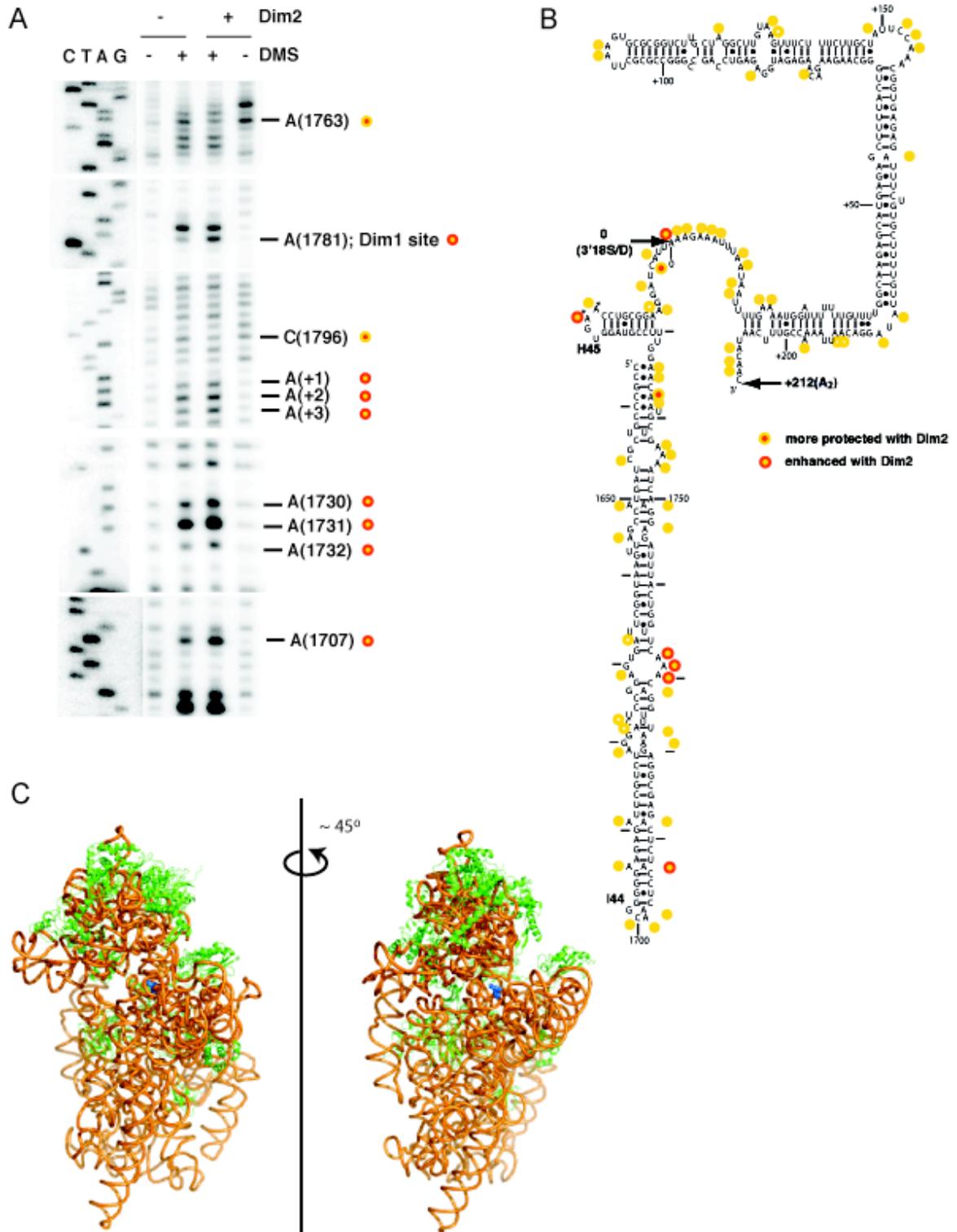


Figure 27: Dim2 binds two sites on rRNA. A) DMS probing revealed methylation of two nucleotides affected by addition of Dim2 indicating that Dim2 binds to these two bases. B) Dim2 binding residues mapped to the secondary structure of the pre-rRNA reveal that one site is located at the top of helix 44 and one between helix 45 and D cleavage site. C) Space fill representation of residue 1763 located at the top of helix 44 shows that the Dim2 binding site is in close proximity to the binding site of Nob1 (reviewed in Chapter 1).

upon binding of Dim2. No sites of protection or enhancements are found within ITS1 (other than directly adjacent to the cleavage site). Importantly, both the sites of protection and enhancement are consistent with our binding data, which indicate that regions between H44 and the 3'-end of 18S rRNA are important for Dim2 binding.

Dim2 Increases Nob1 Affinity for rRNA

As the D-site nuclease, Nob1 binds pre-rRNA containing cleavage site D *in vitro*. We wanted to determine whether Dim2 changes the Nob1•rRNA interaction. We have previously shown that the C-terminal KH domain of Dim2 is required for Dim2 rRNA binding and that the C-terminal KH deletion of Dim2 retains binding to Nob1 (Chapters 2). Because the C-terminal Dim2 deletion does not bind rRNA, we were able to determine a Dim2 affect on Nob1 rRNA binding by adding the C-terminal Dim2 deletion to Nob1 gel-shift mobility RNA binding assays. As shown in Figure 28,

increasing concentrations of the C-terminal deletion of Dim2 results in an increase in Nob1 affinity for rRNA. Since deletion of the C-terminal KH domain abrogates Dim2 rRNA binding the resulting increase in Nob1 rRNA affinity must arise from an allosteric affect of the protein-protein interaction.

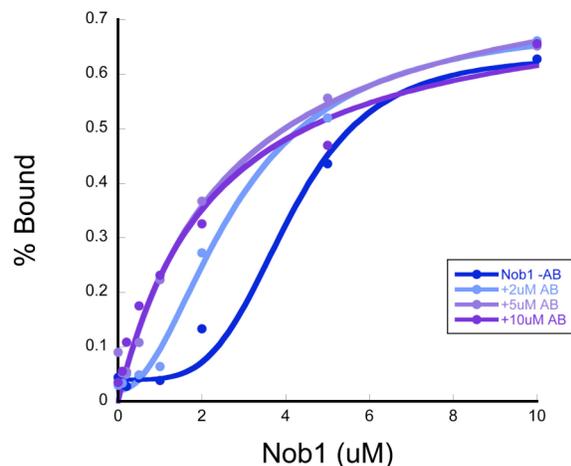


Figure 28: Dim2 increases Nob1 binding to rRNA. Addition of C-terminal KH deletion in Dim2 to Nob1 rRNA binding results in an increase in binding to rRNA due to an allosteric change in Nob1.

Additionally, these data suggest that the Dim2 binding site and the rRNA binding sites of Nob1 interact and Dim2 binding changes the mode of interaction between these two regions of Nob1.

Discussion

We have previously characterized the Dim2 interaction with Nob1, and in this chapter we wanted to further characterize the Dim2 interaction with the rRNA. Based on *in vitro* gel-shift assays and DMS probing, we have shown that Dim2 binds to helix 44 of the rRNA. A second binding site determined by *in vitro* DMS probing is located between helix 44 and helix 45 in a region of rRNA that is single-stranded in pre-A₂ conformation, a conserved sequence of rRNA that is also seen as the binding site of the C-terminal KH domain of aDim2, indicating a conserved binding site for Dim2 from archaea to eukaryotes. Both of these Dim2 binding sites are in close proximity to sites where Nob1 interacts with pre-rRNA, as expected when these proteins directly interact with each other.

While Nob1 cleavage is specific for rRNAs in the post-A₂ cleavage structure, it binds both rRNAs with roughly similar affinities. *In vitro* this leads to some lack in fidelity in that cleavage of the pre-A₂ structure is observed at some frequency, which is enhanced at higher Nob1 concentrations (AC Lamanna & K. Karbstein, unpublished data). In contrast, Dim2 binds stronger to rRNAs in the post-A₂ cleavage conformation. Furthermore, Dim2 also enhances Nob1's RNA binding affinity. This is due to an allosteric effect, as it occurs even with Dim2 mutants that do not bind rRNA by themselves. Therefore, Dim2 could help Nob1

distinguish between rRNA structural conformations to prevent premature cleavage at site A2 and/or promote Nob1-dependent cleavage once the mature pre-rRNA structure has been achieved.

Additionally, based on accessibility to DMS probing, we have shown that Dim2 binding to rRNA changes the rRNA structure. When mapped to helix 44 of mature 18S rRNA, we show that the bases affected by Dim2 binding are present at the internal loop that introduces the kink in helix 44, suggesting that binding of Dim2 could change the kink in helix 44. Interestingly, helix 44 forms many contacts with the large ribosomal subunit, and a substantial number of these are located above the kink. It is possible that the location of these bridges is affected by a kink in helix 44, which could prevent premature association of 60S subunits with assembling cytoplasmic pre-40S ribosomes. Such changes in helix 44 would be akin to changes in its conformation during translation, which have been proposed to, among other things, promote the formation of the hybrid state and translocation.

Further observations show that 1) the binding site of Dim2 involves the same nucleotides as the binding site for bacterial Era, and 2) the binding site of Dim2 is similar to the binding site for RbfA, a bacterial KH domain protein involved in ribosome assembly. These observations point to possible bacterial homologs for Dim2.

Future Directions

To confirm that the residues protected upon Dim2 binding are directly bound to Dim2, future experiments will test whether mutations affect Dim2

binding as expected from a contact. In these experiments rRNA binding will be measured in gel-shift experiments akin to those in Table 3.

Our gel-shift assays have shown that a Dim2 construct lacking the C-terminal KH-domain, does not bind rRNA but increases Nob1 binding to rRNA. We want to know if this increase in Nob1 binding to rRNA is accompanied by a change in the way Nob1 interacts with the rRNA. This will be accomplished in a DMS probing experiment, where the DMS modification pattern of rRNA alone will be compared to that of rRNA in the presence of Nob1 and Nob1 Dim2(Δ C).

Finally, we showed in Chapter II that the D3/K mutation in Dim2 affected Nob1 cleavage. Coupling this mutation with the C-terminal KH domain deletion in Dim2 could test whether the *in vivo* phenotype observed with this mutation is due to a change of Nob1 interaction with rRNA, as determined by DMS probing.

Materials and Methods

Expression and Purification of Dim2: His-MBP-tagged Dim2 plasmid DNA (pSV272; wild type, mutants, and truncations) was freshly transformed into Rosetta cells. Protein expression was carried out as previously described (Lamanna & Karbstein, 2009b). Cells were lysed in 50 mM NaPO₄ (pH 8.0), 150 mM NaCl, and 0.5 mM PMSF and purified via Ni-NTA chromatography using 1ml HisTrap Columns from(GE Healthcare). Ni eluate fractions containing Dim2 were pooled and dialyzed 3 h (or overnight) into 50 mM Tris pH 8.0, 150 mM NaCl, 1mM DTT (Buffer A) at 4°C. Dialyzed protein was loaded onto a MonoS column equilibrated in Buffer A and eluted in a salt gradient to a final concentration of 40% of Buffer B (50 mM Tris pH 8.0, 2 M NaCl, 1 mM DTT) over 12 column

volumes. Fractions containing Dim2 were pooled and concentrated and further purified over a Superdex200 column in 50mM HEPES pH 8.0, 200 mM NaCl, 1mM DTT. Dim2-containing fractions were pooled and concentrated and flash frozen and stored at -80°C. Protein concentration was determined with a nanodrop spectrophotometer using $A=\epsilon lc$.

Expression and Purification of Nob1: His-MBP-tagged Nob1 plasmid DNA (pSV272; wild type, mutants, and truncations) was freshly transformed into Rosetta cells. Protein expression was carried out as previously described (Lamanna & Karbstein, 2009). Cells were lysed in 50 mM NaPO₄ (pH 8.0), 150 mM NaCl, and 0.5 mM PMSF and purified via Ni-NTA chromatography. Ni eluate fractions containing Nob1 were pooled and dialyzed 3 h (or overnight) into 50 mM Tris pH 8.0, 150 mM NaCl, 1mM DTT (Buffer A) at 4°C. Dialyzed protein was loaded onto a MonoS column equilibrated in Buffer A and eluted in a salt gradient to a final concentration of 40% of Buffer B (50 mM Tris pH 8.0, 2 M NaCl, 1 mM DTT) over 12 column volumes. Fractions containing full-length Nob1 were pooled and concentrated and further purified over a Superdex200 column in 50mM HEPES pH 8.0, 200 mM NaCl, 1 mM DTT. Nob1-containing fractions were pooled and concentrated and flash frozen and stored at -80°C. Protein concentration was determined with a nanodrop spectrophotometer using $A=\epsilon lc$.

Transcriptions: rDNA fragments were cloned into pUC19 between XmaI and PstI sites as described (Lamanna & Karbstein, 2009). 50 mL overnight cultures were

grown in LB medium with Ampicillin and plasmid DNA isolated using a maxiprep kit (Qiagen). Plasmid DNA was digested with BtsI (NEB), phenol: chloroform extracted and resuspended in 200 μ L of water. Transcriptions were carried out as described (Lamanna & Karbstein, 2009) using the entire digested rDNA template in a final volume of 5 mL. The reaction was terminated by adding 0.5 mL of 0.5 M EDTA (pH 8.0), and RNA was precipitated overnight at -20°C by adding 0.55 mL of 3 M sodium acetate and 16.5 mL ethanol. RNA was purified using an RNA Maxi Kit (Qiagen). To obtain body-labeled transcripts reactions were carried out as previously described (Lamanna & Karbstein, 2009a).

RNA Binding Assay: RNA was folded as described (Lamanna & Karbstein, 2009b). Dim2 was pre-incubated at 30°C for 15-20 minutes before being RNA was added to binding tubes. 10 μ l binding reactions were incubated at 30°C for 2 hours and then immediately loaded onto an 8% Acrylamide/THEM pH 8.0 gels (33 mM Tris, 67 mM HEPES, 1 mM EDTA, 10 mM MgCl_2) after addition of 2 μ l of RNA loading dye (0.1% xylene cyanol, 0.1% bromophenol blue, 45% glycerol). Gels were run at 250V for \sim 2 hours, dried and exposed to a phosphoscreen overnight. Signal was detected using Typhoon scanner and quantification was performed using ImageQuant software.

References

- Bohnsack, M. T., Martin, R., Granneman, S., Ruprecht, M., Schleiff, E., & Tollervey, D. (2009). Prp43 bound at different sites on the pre-rRNA performs distinct functions in ribosome synthesis. *Molecular Cell*, *36*(4), 583-592.
doi:10.1016/j.molcel.2009.09.039
- Fatica, A., Tollervey, D., & Dlakic, M. (2004). PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. *RNA (New York, N.Y.)*, *10*(11), 1698-1701.
doi:10.1261/rna.7123504
- Granneman, S., Kudla, G., Petfalski, E., & Tollervey, D. (2009). Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(24), 9613-9618.
doi:10.1073/pnas.0901997106
- Granneman, S., Petfalski, E., Swiatkowska, A., & Tollervey, D. (2010). Cracking pre-40S ribosomal subunit structure by systematic analyses of RNA-protein cross-linking. *The EMBO Journal*, *29*(12), 2026-2036.
doi:10.1038/emboj.2010.86
- Jia, M. Z., Ohtsuka, J., Lee, W. C., Nagata, K., & Tanokura, M. (2007). Crystal structure of Dim2p: A preribosomal RNA processing factor, from *pyrococcus horikoshii* OT3 at 2.30 Å. *Proteins*, *69*(2), 428-432. doi:10.1002/prot.21381
- Lamanna, A. C., & Karbstein, K. (2009a). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings*

of the National Academy of Sciences of the United States of America,
106(34), 14259-14264. doi:10.1073/pnas.0905403106

Lamanna, A. C., & Karbstein, K. (2009b). Nob1 binds the single-stranded cleavage site D at the 3'-end of 18S rRNA with its PIN domain. *Proceedings of the National Academy of Sciences of the United States of America*, 106(34), 14259-14264. doi:10.1073/pnas.0905403106

Vanrobays, E., Gelugne, J. P., Caizergues-Ferrer, M., & Lafontaine, D. L. (2004). Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. *RNA (New York, N.Y.)*, 10(4), 645-656.

Vanrobays, E., Leplus, A., Osheim, Y. N., Beyer, A. L., Wacheul, L., & Lafontaine, D. L. (2008). TOR regulates the subcellular distribution of DIM2, a KH domain protein required for cotranscriptional ribosome assembly and pre-40S ribosome export. *RNA (New York, N.Y.)*, 14(10), 2061-2073.
doi:10.1261/rna.1176708