RNP REMODELING AND COFACTOR MODULATION BY THE DEAD-BOX PROTEIN DED1P

by

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Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

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(date) September 22, 2008
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>adenosine 5’ (beta, gamma-imido) triphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ded1</td>
<td>defines essential domain 1</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>(ethylenedinitrilo)-tetra acetic acid</td>
</tr>
<tr>
<td>eIF4A</td>
<td>eukaryotic Initiation Factor 4A</td>
</tr>
<tr>
<td>EJC</td>
<td>exon junction complex</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid</td>
</tr>
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<tr>
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</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside 5’-triphosphate</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tryptophan RNA-binding attenuation protein</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>WT</td>
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<tr>
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RNP Remodeling and Cofactor Modulation by the DEAD-box Protein Ded1p

ABSTRACT

by

HEATH BOWERS

DEAD-box proteins are involved in virtually all aspects of eukaryotic RNA metabolism (3). As members of the helicase superfamily 2 (SF2), DEAD-box proteins utilize ATP hydrolysis to unwind RNA, assemble large protein complexes on RNA, and remodel RNA protein complexes (RNPs) (4-6). In the cell however, DEAD-box proteins function in the context of RNPs during processes such as splicing and translation (5, 6). How the biochemical activities of DEAD-box proteins are utilized in a physiological setting is an important and central question in RNA metabolism. In this thesis, we address this issue by examining RNP remodeling and cofactor modulation by the DEAD-box protein Ded1p.

We demonstrated that Ded1p did not actively displace the RNA binding proteins U1A and TRAP from their cognate RNA binding sites. Additionally, we established that the context of a RNP determined active displacement by Ded1p and propose a model for RNP remodeling by DEAD-box proteins. We found that an inability to actively displace other proteins from RNA can provide non-sequence specific DEAD-box proteins with the capacity to disassemble similar RNA complexes in a discriminatory fashion.
We further identified a physiologically relevant interaction between Ded1p and the translation initiation factor eIF4G. We showed that eIF4G did not modulate Ded1p ATPase activity, but did inhibit strand separation by Ded1p. Interestingly, Ded1p greatly increased eIF4G’s affinity for RNA even to RNAs too small for eIF4G to bind alone. Our results suggest that Ded1p’s biochemical activities facilitate eIF4G RNA binding. We propose a basic model for Ded1p enhancement of eIF4G RNA binding which may be relevant for Ded1p’s role during translation initiation.
CHAPTER 1: GENERAL INTRODUCTION TO DEAD-BOX PROTEINS

1.1: OVERVIEW

DEAD-box proteins are the largest group of enzymes in eukaryotic RNA metabolism with roles in transcription, ribosome biogenesis, mRNA splicing, translation, and RNA turnover (7-12). Defined by nine highly conserved sequence motifs (13, 14), DEAD-box proteins are members of the large superfamily 2 (SF2) (14). SF2 helicases utilize the energy from ATP to unwind RNA and remodel or assemble ribonucleoprotein (RNP) complexes (14, 15). Advances have been made in understanding the biochemical activities and mechanisms of DEAD-box proteins (16), but how these activities function in their cellular context or how they are regulated is not well understood. In this thesis, we examine two functional aspects of DEAD-box proteins, RNP remodeling and cofactor regulation.

In the cell, DEAD-box proteins are often part of large multi-component assemblies, such as the splicesome or the translation initiation machinery (5, 6), and it is of particular interest to understand how DEAD-box proteins function in such environments. To solve this problem, we examine here how the DEAD-box protein Ded1p from *Saccharomyces cerevisiae* remodels RNPs and investigate how Ded1p and a physiological cofactor, eIF4G, affect each other’s biochemical function.

1.2: DEAD-BOX PROTEINS

DEAD-box proteins belong to the helicase superfamily II (SF2), which is a subset of the P-Loop NTPases (Figure 1.1). DEAD-box proteins contain at least nine characteristic
sequence motifs that are conserved from bacteria to man (Figure 1.2) (13, 14). Motifs I and II are the walker A and B motifs featured by P-Loop proteins. Both motifs are critical for ATP binding and hydrolysis (14). The Q-motif confers the ATP specificity for DEAD-box proteins through contacts with the adenine ring of ATP (2). Motifs III and VI are thought to coordinate ATP hydrolysis with helicase function (14). Motifs Ia, Ib, IV, and V are involved in RNA binding (14).

Crystal structures of DEAD-box proteins revealed that the core motor of DEAD-box proteins resembles a dumbbell shape consisting of two RecA-like domains that are connected by a linker region (Figure 1.3) (2). All nine characteristic sequence motifs lie within the core domain (2, 14).

The catalytic core of DEAD-box proteins resembles that of SF1 and other SF2 helicases (14). In addition, DEAD-box proteins and other SF2 helicases share many of the same conserved motifs (14). However, despite structural and sequence similarities between canonical SF2 helicases and DEAD-box proteins, DEAD-box proteins unwind RNA by a distinct mechanism (17, 18). Most studied helicases translocate on the nucleic acid often taking multiple steps along the substrate before dissociating (19). As the helicase translocates, the enzyme displaces whatever is bound to the nucleic acid, whether it is another strand of nucleic acid or a RNA-binding protein (Figure 1.4.A) (16, 19). Canonical SF2 helicases also have a defined unwinding polarity; that is the helicase travels unidirectionally from one end of the nucleic acid to the opposite end (16, 19).
Figure 1.1. Evolutionary relationship of RNA helicases to other P-loop “motors.” Figure was adapted from reference (1). Phylogenetic tree for selected classes of P-loop proteins. The trees for the P-loop proteins were constructed based on the topology (black tree) or RMSD values (lavender) of the ATP binding domains. Topology diagrams under the protein classes indicate β-strands (triangles) and α-helices (circles), arrows denote additional C-terminal motifs, double hatches indicate additional domains. Walker A is colored red, Walker B is colored orange. In the protein structures, the ATP binding domain is colored blue, additional domains as gray ribbons. Walker A and B are colored as above. The phylogenetic tree for SF2 helicases was calculated based on sequence comparison of RNA helicases. In the helicase structures the P-loop containing ATP binding domain is colored blue. The second “helicase domain” (Fig. 1B, C) is represented by light blue ribbons, other domains are shown as gray ribbons. Walker A and B are colored as above. Structures shown: G-proteins: Ras (pdb ID: 4Q21), Kinesin (3KIN), Myosin (1BR1), F1ATPase (1BMF), ABC Transporter: His Permease (1BOU), AAA Protein: Hslu Protease (1G3I), SF1 Helicase: PcrA (2PJR), RecQ (1OYW), DExH: HCV NS3 (1HEI), DEAD: mjDEAD (1HV8), and SF3 Helicases: Rep40 (1S9H).
**Figure 1.2.** Conserved sequence motifs of DEAD-box proteins. The names of the conserved are labeled on the top. The letters in the squared box represent the consensus sequences of respective motifs in the single-letter amino acid code and x represents any amino acid residue. Red boxes, motifs involved in NTP binding and hydrolysis; blue, RNA binding; gold, coupling of ATP binding and hydrolysis to duplex unwinding; purple, conferring ATP specificity in the DEAD-box subgroup. Upper case represents, >75% conserved within each DExH/D helicases subgroup; lower case represents, >50% conserved. The figure is adapted from reference (2).
Figure 1.3. Structural characteristics of DEAD-box proteins. Side (left) and front (right) views of the DEAD-box helicase VASA in complex with RNA and the non-hydrolysable ATP analog AMPPNP. Bound RNA is colored in green. AMPPNP is shown in a ball-and-stick representation. Locations of conserved sequence motifs are labeled in different colors. The figure is adapted from reference (2).
This polarity is reflected in the requirement for a single stranded (ss) region at one defined orientation relative to the duplex. Helicases that unwind 3’ to 5’ require the ss region at the 3’ end, while helicases that unwind 5’ to 3’ require the ss region at the 5’ end.

DEAD-box proteins, however, do not translocate and thus do not have a defined polarity (17). To unwind RNA duplexes, DEAD-box proteins bind directly to the duplex region and then pry the strands apart in an ATP-dependent fashion (Figure 1.4.B) (17). Single stranded regions facilitate loading of DEAD-box proteins to the duplex, but the orientation of the ss region is not important (18).

1.3: PHYSIOLOGICAL ROLES AND FUNCTIONS OF DEAD-BOX PROTEINS.

DEAD-box proteins are involved in a variety of RNA-related processes, including transcription, ribosome biogenesis, pre-mRNA splicing, RNA export, translation, and RNA degradation (9, 10, 12, 20-23). Within these processes, DEAD-box proteins are believed to act in highly specific steps, and are thought to function to unwind small patches of RNA, assemble large RNPs, or rearrange RNPs (4-6).

An example of a DEAD-box protein that rearranges small basepaired regions of RNA is Dbp4p (4). Dbp4p functions during maturation of the 90S ribosomal RNA (rRNA) to remove the U14 small nucleolar RNA (snoRNA) (4). U14 snoRNA basepairs to complementary sequences within the 90S rRNA (24).
Figure 1.4. Unwinding mechanism of typical SF2 and DEAD-box helicases. (A) Schematic representing the unwinding mechanism of processive unidirectional helicases. The lines represent a RNA duplex. (B) Schematic representing the unwinding mechanism of DEAD-box proteins. The lines represent a RNA duplex.
Deletion of Dbp4p from yeast dramatically increased U14 snoRNA association with preribosomes (4). Furthermore, Dbp4p mutations in motifs I and III, both of which are important for ATP hydrolysis and helicase activity, blocked release of U14 snoRNA from pre-ribosomes, indicating that Dbp4p is required to unwind the duplex between U14 snoRNA and 90S rRNA (4).

DEAD-box proteins can also function to assemble RNPs. The DEAD-box protein eIF4AIII plays a variety of roles in post-processing mRNA metabolism, including nonsense-mediated decay and translation (25). eIF4A is a component of the Exon Junction Complex (EJC), which is deposited on mRNA ~20 nt upstream of 5’ exons during mRNA splicing (26). Once bound to the mRNA, proteins within the EJC inhibit the ATP hydrolysis activity of eIF4AIII thereby locking eIF4AIII onto the mRNA (27). Within the EJC, eIF4AIII acts like an ATP-dependent RNA clamp (28).

Although examples of DEAD-box proteins unwinding RNA-RNA interactions and assembling RNPs exist, it is widely believed that the vast majority of DEAD-box proteins function as RNPases; that is, enzymes that remove protein complexes from RNA (15). In several cases, DEAD-box proteins are essential for the removal of protein from RNA, as discussed below.

1.4: RNPs REMODELED BY DEAD-BOX PROTEINS
The first specific examples of DEAD-box proteins targeting RNA–protein interactions emerged for Prp28p, Sub2p, Prp5p and Dbp5p (6, 20, 22, 29). Dbp5p is involved in
mRNA export (30), and the other three proteins are essential components of the pre-
mRNA splicing machinery (31). The DEAD-box protein Prp28p is involved in
exchanging U1snRNA with U6snRNA on the 5’ splice site and has been implicated in the
removal of the U1snRNP from the 5’ splice site (32). The U1snRNP binds to the 5’
splice site by forming a short RNA–RNA helix that is stabilized through several RNA-
protein interactions (33, 34). One of these stabilizing proteins is U1Cp, and Prp28 is
thought to counteract the stabilizing effect of U1Cp (20, 35). If U1Cp contains a mutation
that reduces its affinity for the RNA, the otherwise essential Prp28p becomes
dispensable, suggesting that Prp28p is responsible for the removal of U1Cp (20).

A similar bypass suppressor strategy illuminated the involvement of the DEAD-box
protein Sub2p in the displacement of the protein Mud2p (29). Among other functions,
which include a role in RNA export (36), Sub2p participates in early spliceosome
assembly by promoting the exchange of the branch point-binding protein (BBP) with the
U2 snRNP at the pre-mRNA branch site (8). The binding of BBPp to the branch site is
presumably stabilized by the non-essential protein Mud2p (37). Deletion of Mud2
obviates the need for the essential Sub2p, consistent with a role of Sub2p in Mud2p
displacement (29).

Prp5p is required for the stable addition of the U2 snRNP to the branch site, which
normally depends on the ATPase activity of Prp5p (38). However, the interaction of the
U2 snRNP with the branch site can also occur with ATPase-deficient Prp5p, but only if
the non-essential protein Cus2p is deleted (6). Thus, the essential Prp5p cannot be
completely bypassed by deletion of Cus2p. Nonetheless, the normally essential ATPase activity of Prp5p can be made obsolete (6), suggesting that Prp5p dislodges Cus2p in an ATP-dependent manner.

Recently, it has been shown that the DEAD-box protein Dbp5p, which functions in late steps of mRNA export on the cytoplasmic side of the nuclear pore complex, is also likely to be specifically required for displacement of the protein Mex67 from RNA (22). It was shown that in dbp5 mutant cells, the mRNA export receptor Mex67 accumulates on mRNA, and that these Mex67-bound RNAs were enriched at the nuclear rim (22). The accumulation of Mex67-bound RNAs in dbp5 mutant cells was suppressed by a mex67 mutation, consistent with a scenario where Dbp5p removes Mex67 from the RNA.

Although the observations for Prp28p, Sub2p, Prp5p and Dbp5p strongly suggested the involvement of these enzymes in the removal of other proteins from RNA, the mechanisms by which DEAD-box enzymes caused protein displacements remained unclear (31). For example, the data obtained with Prp28p, Sub2p, Prp5p and Dbp5 did not illuminate whether DEAD-box proteins rely on other cofactors or on a specific context to displace proteins, or whether DEAD-box proteins alone are sufficient to dislodge other proteins. It also remained unclear whether DEAD-box proteins are able to act directly on the respective RNA–protein interaction, or whether the enzymes displace proteins only indirectly, possibly through the remodeling of RNA secondary structure. These questions are addressed in this thesis.
1.5: RNP REMODELING WITH MODEL SUBSTRATES

To elucidate the basic mechanism(s) of protein displacement by DEAD-box proteins, it is critical to quantitatively analyze RNP remodeling reactions. Since it is unknown precisely where the vast majority of DEAD-box proteins bind to their targets and which exact conformational changes the enzymes catalyze in their respective substrates (14, 39), it has not yet been possible to devise model systems that recapitulate a physiological RNP remodeling reaction that can be analyzed quantitatively as well. For example, complex in vitro systems such as pre-mRNA splicing extracts provide invaluable qualitative information about DEAD-box protein function, but the limited control over parameters such as concentrations of individual factors currently precludes the use of these systems for quantitative mechanistic studies. Thus to date, it is only possible to obtain quantitative mechanistic information about RNP remodeling by DEAD-box proteins with simple, yet non-physiological RNP models.

While removal of proteins from model RNPs had yet to be demonstrated for DEAD-box proteins at the start of this thesis, displacement of the RNA binding protein U1A from its cognate RNA binding site in a model RNP had been shown by the DExH protein NPH-II (40). DExH proteins also belong to the helicase SF2 (19). NPH-II is a processive RNA helicase involved in replication of Vaccinia virus (19). U1A is part of the pre-mRNA splicing machinery, and it also acts as a feedback inhibitor for its own gene expression (41, 42). In the model RNP, U1A forms a homo-dimer on the RNA, contacting predominantly the single-stranded loops embedded in the helical regions of the RNA (43).
NPH-II increased the dissociation rate constant of U1A from the RNA by more than three orders of magnitude in an ATP-dependent fashion (40). That is, NPH-II did not ‘wait’ until U1A dissociated spontaneously to then unwind the U1A-binding site; rather, NPH-II actively displaced U1A (40). Further kinetic analysis of the RNP remodeling showed that U1A displacement was in fact faster than RNA unwinding by NPH-II. The processivity of NPH-II was decreased, but not completely eliminated by the U1A displacement event, i.e. the enzyme could continue to unwind RNA duplexes after dislodging U1A without first leaving the RNA (40).

1.6: COFACTOR MODULATION OF DEAD-BOX PROTEINS

The biochemical characterization of DEAD-box proteins has been conducted with minimal model systems consisting of a DEAD-box protein and a RNA substrate. Control over parameters such as concentration allows for quantitative measurements that lead to insight into the mechanism of DEAD-box proteins. In the cell, however, DEAD-box proteins function in the context of larger protein assemblies during biological processes like mRNA splicing, ribosome biogenesis, and translation (9, 21, 44) and thus interact with other proteins. The protein-protein and RNA-protein contacts made between the protein assemblies and DEAD-box proteins can affect the RNA binding, ATPase, and helicase activities of DEAD-box proteins (5, 12). Since it is still difficult to discern which proteins modulate DEAD-box proteins within a large complex, studies have focused on proteins known to interact with DEAD-box proteins to determine how they affect the functions of the helicases (Table 1.1).
Proteins that bind to and affect the RNA binding, ATPase, or helicase activities of DEAD-box proteins are generally referred to as helicase cofactors (45). One of the best studied examples of helicase modulation by a cofactor is the DEAD-box protein eIF4A1 and the translation initiation factor eIF4B. eIF4A1 functions in translation initiation as a component of the larger protein complex eIF4F (46). eIF4B contains a RNA recognition motif (RRM) (47) and has been genetically linked to eIF4A1 (48). eIF4B improved the RNA binding, ATPase, and helicase activities of eIF4A1 when present (44, 49). These studies revealed that a cofactor-dependent increase in the RNA affinity for a DEAD-box protein can result in stimulated ATP hydrolysis and strand separation (44).

Despite the observations made with eIF4A and eIF4B, helicase cofactors do not always enhance the enzymatic activities of DEAD-box proteins. The DEAD-box protein eIF4AIII and Magoh-Y14 are components of the exon junction complex (EJC) (26). When RNA binding was examined in the presence of Magoh-Y14, eIF4AIII bound RNA tighter and did not dissociate (27). The increased RNA binding did not result in stimulated ATP hydrolysis as would be expected from the example of eIF4A1 and eIF4B. Instead, the ATPase activity of eIF4AIII was inhibited by Magoh-Y14 (5, 27). The inhibition of ATP hydrolysis enables the DEAD-box protein to serve as an RNA anchor for the EJC. Crystal structures revealed that eIF4AIII ATPase activity is inhibited because Magoh-Y14 locks eIF4AIII into the RNA bound conformation, preventing eIF4AIII from undergoing the necessary conformational changes to either enable hydrolysis of ATP or to release the inorganic phosphate (28). These results demonstrated
that cofactors can add additional function to DEAD-box proteins by inhibiting the enzyme.

<table>
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<tr>
<th>Helicase</th>
<th>Cofactor</th>
<th>RNA binding</th>
<th>ATPase</th>
<th>helicase</th>
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<tr>
<td>Dbp5p</td>
<td>InsP6/Gle1p</td>
<td>nd.\textsuperscript{a}</td>
<td>+</td>
<td>nd.\textsuperscript{a}</td>
<td>(50)</td>
</tr>
<tr>
<td>Dbp8p</td>
<td>Ess2p</td>
<td>nd.\textsuperscript{a}</td>
<td>+</td>
<td>nd.\textsuperscript{a}</td>
<td>(51)</td>
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<tr>
<td>elf4B</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(44, 49, 52)</td>
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<tr>
<td>elf4I</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(49, 52-54)</td>
</tr>
<tr>
<td>elf4G</td>
<td></td>
<td>+</td>
<td>+</td>
<td>nd.\textsuperscript{a}</td>
<td>(44, 55)</td>
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<tr>
<td>elf4AII</td>
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<td>+</td>
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<td>(5, 27)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>(56, 57)</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>nd.\textsuperscript{a}</td>
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<td></td>
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<td>nd.\textsuperscript{a}</td>
<td>no effect</td>
<td>no effect\textsuperscript{c}</td>
<td>(59)</td>
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(a) Not Determined.
(b) As determined by (60)
(c) As determined by (59)

As discussed, cofactors can stimulate or inhibit the enzymatic activities of DEAD-box proteins, but other proteins that interact with DEAD-box proteins do not always affect their function. An example is the interaction between the DEAD-box protein UAP56 and the splicing factor U2AF\textsuperscript{65}. UAP56 was originally identified through its interaction with U2AF\textsuperscript{65} (21). UAP56 is required for multiple steps during spliceosomal assembly (21, 61) and for efficient export of mRNAs from the nucleus (62). U2AF\textsuperscript{65} binds and stabilizes the branch binding protein (BBP) on the pre-mRNA during splicing (63). When tested, U2AF\textsuperscript{65} did not enhance or inhibit the ATPase or helicase activity of UAP56 (59).
1.7: THE DEAD-BOX PROTEIN Ded1p

To gain mechanistic insight into RNP remodeling by and cofactor modulation of DEAD-box proteins, we examined the DEAD-box protein Ded1p \textit{in vitro}. We chose to study the DEAD-box protein Ded1p because a basic mechanism for unwinding has been established (17), and Ded1p’s biochemical activities are well characterized (17, 18, 64, 65).

Ded1p (defines essential domain 1) was originally identified as an essential open reading frame during the mapping of the \textit{S. cerevisiae} genome (66). Ded1p contains the nine conserved sequence motifs characteristic for a DEAD-box protein, an arginine-glycine-aspartagine rich C- and N-termini (64, 67), and a conserved N-terminal nuclear export signal (NES) (68). Ded1p has homologs and orthologs in the higher organisms (69), including Belle in \textit{Drosophila melanogaster} (70), An3 in \textit{Xenopus laevis} (71), PL10 in mice (72), and DDX3 and DBY in human (73, 74). Ded1p is also closely related to Dbp2 in \textit{S. cerevisiae}, VASA in \textit{X. laevis}, and DDX4 and p68 in human (69, 75). Notably, DDX3 from human, as well as Belle from \textit{D. melanogaster}, can rescue the lethality of a DED1 null mutation in yeast but not \textit{vise versa} (70, 76).

Ded1p, like many DEAD-box proteins, functions in several processes of RNA metabolism (77). Ded1p was first implicated in pre-mRNA splicing (78). Overexpression of Ded1p suppressed a temperature sensitive growth defect caused by a mutation in the splicing factor Prp8p (78). Later studies found that Ded1p was a component of the large spliceosomal complex, the penta-snRNP (79). In addition, it was
demonstrated that Ded1p associated with the U1, U2, U5, and U6 small nuclear ribonucleoproteins (snRNPs), and that mutations in Ded1p caused splicing defects (80).

Ded1p was also isolated with pre-ribosomal particles implicating Ded1p in another nuclear RNA processing event, ribosome biogenesis (81). The ortholog of Ded1p from *X. laevis*, An3, localizes during development to the nucleoli, the compartment where rRNA biogenesis occurs (82). The majority of the rRNAs are transcribed by RNA polymerase I while RNA polymerase III is responsible for transcribing the small 5S rRNA. Interestingly, overexpression of Ded1p was found to suppress a C-terminal deletion mutation in RNA polymerase III (83).

Ded1p also plays a role in RNA export from the nucleus. Ded1p and its orthologs have a conserved nuclear export signal (NES) that is utilized by Crm1p for export (68, 84). The Crm1p export pathway is an essential RNA shuttling pathway (85). In addition, Ded1p interacts with another RNA shuttling protein, Npl3p (86). Helicase activity is important for the export of RNA out of the nucleus as the Ded1p ortholog An3 and mRNA accumulated in the nucleus when a mutation disrupted the helicase activity of An3 (87).

Apart from its nuclear roles, Ded1p is required for translation initiation in the cytoplasm (23). Depletion of Ded1p in an *in vitro* yeast translation system abolishes translation and this defect can be rescued by addition of recombinant Ded1p (23). In addition, the fraction of translating ribosomes decreases when yeast harboring a cold sensitive Ded1p mutation was shifted to the nonpermissive temperature, suggesting a defect in translation.
initiation (23). Ded1p also localizes to P-bodies during translational repression (88). P-bodies are a cluster of untranslating mRNAs assembled into large mRNPs where the mRNAs can either be degraded or returned to translation (89). Overexpression of Ded1p results in increased size and number of P-bodies as well as slowed growth (88), indicating that Ded1p may play a role in enhancing and repressing translation.

The biochemical activities of Ded1p are well characterized. Ded1p possesses a very weak basal ATPase activity that is stimulated when Ded1p binds RNA (65). Ded1p, like other DEAD-box proteins, uses ATP to unwind RNA duplexes using a local strand separation mechanism (17, 18). In addition, Ded1p can anneal two complementary strands of RNA in an ATP-independent fashion (64). Ded1p can also facilitate more complex RNA structure conversions (90).

How Ded1p and other DEAD-box proteins use these activities in their physiological environment is not understood. For example, if Ded1p can displace proteins from RNA, does it do so using a mechanism similar to unwinding? Additionally, are there cofactors that stimulate or inhibit the well-characterized biochemical activities of Ded1p? The results presented in this thesis address these questions.
CHAPTER 2: RNP REMODELING BY Ded1p

2.1: INTRODUCTION

While DEAD-box proteins are required to unwind RNA duplexes and assemble RNPs (4, 5), the majority of DEAD-box proteins are believed to function in the removal of proteins from RNA (15, 31). Notwithstanding, direct displacement of a RNA binding protein by a DEAD-box protein had yet to be shown. However, the DExH protein NPH-II actively displaced the RNA binding protein U1A from its cognate RNA binding site (i.e. displacement of U1A occurs faster than spontaneous dissociation of U1A from the RNA) (40). Using NPH-II as a standard of comparison for helicase-mediated protein displacement from RNA, we can begin to investigate RNP remodeling by DEAD-box proteins. To approach this issue, we first asked whether DEAD-box proteins actively displace proteins from RNA.

2.2: RESULTS

2.2.1: U1A BASED SUBSTRATES

We first tested whether Ded1p, like NPH-II, could displace U1A from its cognate RNA. To this end, we created a substrate containing a U1A binding site derived from the 3’-untranslated region (UTR) of the U1A mRNA (Figure 2.1.A). U1A binds this RNA as a homodimer interacting with the single-stranded loops that are embedded into helical structures (Figure 2.1.A&B) (43). A second substrate utilizing this duplex with a 24 nt long single-stranded region appended to the 3’ end of the helix was also used. This single stranded region provides a binding site for the helicase on the RNP.
We first determined the binding characteristics of U1A to these RNA substrates. Under our reaction conditions, U1A bound to the RNA duplex (Figure 2.2.A) and the RNA duplex with the 3’ 25nt ssRNA with apparent dissociation constants of $K_D = 1.7 \pm 0.5$ nM and $K_D = 5.1 \pm 0.5$ nM respectively (Figure 2.2.B). U1A dissociated from both model substrates in a biphasic reaction (Figure 2.2.C). The biphasic dissociation kinetics of U1A, which was observed with different U1A preparations, was independent of the U1A concentration, and changes in the reaction conditions did not alter the biphasic shape of the dissociation time course (data not shown). For these reasons, the biphasic dissociation kinetics of U1A is likely to reflect either inherent heterogeneity in the U1A–RNA complex or induced fit binding of U1A to the RNA (91, 92).

2.2.2: Ded1p REMODELING OF U1A BASED SUBSTRATES

To test whether Ded1p could displace U1A from the RNA, we measured disassembly of the U1A–RNA complex by following the separation of the two RNA strands. In the presence of U1A, strand separation indicates protein removal as previously shown with NPH-II (40). To measure U1A displacement by Ded1p, RNA substrates with or without U1A were incubated with Ded1p. ATP was added to start the reactions, aliquots were removed, and the reaction was stopped with SDS and EDTA. The aliquots were then loaded onto a non-denaturing PAGE. These experiments revealed that Ded1p readily unwound both RNA complexes in the absence of U1A (Figure 2.3.A, 2.3.B). However, in stark contrast to previously published observations with NPH-II (40, 93), Ded1p did not efficiently disassemble the RNA strands with U1A bound on either RNA substrate (Figure 2.3.A, 2.3.B).
**Figure 2.1**

(A) RNP design. Sequence of the RNA strands. U1A binds to the single-stranded loops as depicted by gray ovals.

(B) Structure of the U1A RNA complex as determined by NMR. Varani et al. *Nat.Struct.Biol.* 7 pp. 329 (2000)
Figure 2.2

A

B

C

U1A – +

Fraction Bound

U1A [nM]

Fraction Free

Time [min]

Fraction Free

Time [min]
FIGURE 2.2. U1A based RNP. (A) U1A binding to the substrate without a 3’ 25nt ssRNA. Radiolabeled RNA complex (0.5 nM) was incubated without (left lane) and with 20 nM U1A (right lane) under reaction conditions for 10 minutes and applied to 8% non-denaturing PAGE. The cartoons indicate free and U1A (oval) bound RNA complexes. (B) Equilibrium binding of U1A to the RNA duplex without (filled circle) and with (open circle) a 3’ 25nt ssRNA. Data points represent the average of at least three independent measurements. Error bars represent one standard deviation. Data were fit to the Hill-equation ($K_D^{\text{filled}} = 1.7 \pm 0.5 \text{ nM}, n = 1.9 \pm 0.2$; $K_D^{\text{open}} = 5.1 \pm 0.5 \text{ nM}, n = 1.4 \pm 0.1$). (C) Spontaneous dissociation of U1A from the RNA without (filled circles) and with (open circles) a 3’ 25nt ssRNA. The representative time courses were fit to the sum of two exponentials (dissociation rate constants were, for the first phase: $k_d^{\text{filled}} = 7.3 \pm 0.0 \text{ min}^{-1}$, and for the second phase: $k_d^{\text{filled}} = (2.5 \pm 4.4) \times 10^{-3} \text{ min}^{-1}$); and $k_d^{\text{open}} = 0.24 \pm 0.1 \text{ min}^{-1}$, and for the second phase: $k_d^{\text{open}} = (1.8 \pm 0.2) \times 10^{-3} \text{ min}^{-1}$).
To further understand these dissimilarities between NPH-II and Ded1p, we measured the kinetics of the RNA strand separation with and without bound U1A (Figure 2.3.C, 2.3.D). Without U1A, Ded1p readily unwound the RNA duplex and RNA duplex with a 3’ 25nt ssRNA with apparent first order rate constants of $k_{\text{unw}} = 0.8 \pm 0.1$ min$^{-1}$ and $k_{\text{unw}} = 0.9 \pm 0.1$ min$^{-1}$, respectively (Figure 2.3.C, 2.3.D). With U1A bound, however the time course for strand separation strikingly resembled the biphasic kinetics of the spontaneous U1A dissociation (Figure 2.3.C, 2.3.D, cf. Figure 2.2.C). Apparently, Ded1p did not accelerate the dissociation of U1A from the RNA. The enzyme could only separate the RNA strands upon spontaneous dissociation of U1A. This phenomenon was independent of the presence of a 25nt ssRNA region appended to the 3’ end of the duplex.

To verify that the inability of Ded1p to actively displace U1A was not due to sub-saturating ATP or Ded1p concentrations, we measured the kinetics of U1A remodeling under various concentrations of ATP and Ded1p. The data from the reactions were fit to a sum of 2 exponentials. To illustrate remodeling efficiency as a function of ATPase and Ded1p concentration, the amplitude of the first exponential was plotted against either ATP or Ded1p concentrations (Figure 2.4). With increasing ATP concentration, the first amplitude increased in a hyperbolic manner, until the maximal amplitude reached 0.23 (Figure 2.4.A). For increasing Ded1p concentrations, we observed a sigmodial increase of the amplitude of the first exponential. A plateau was reached at a Ded1p concentration higher than $\sim$200 nM (Figure 2.4B). Together, these data suggest that the lack of active displacement was not due to sub-saturating ATP and Ded1p concentrations.
Figure 2.3

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C

D
Figure 2.3. Ded1p does not actively displace U1A from RNA without (A) or with (B) a 3’ 25nt ssRNA region (representative PAGE). Mobilities of the RNA complex and the single-stranded RNA are indicated by cartoons on the left. Reactions were allowed to proceed for 5 min. (C) Time course of Ded1p-catalyzed unwinding of the RNA without a 3’ 25nt ssRNA complex with and without U1A bound. RNA strand separation by Ded1p with U1A bound (open circles) and in the absence of U1A (filled circles). The resulting time course for the reaction without U1A was fit against the integrated rate law for a homogenous first-order process, yielding an unwinding rate constant of $k_r^{[RNA]} = 0.83 \pm 0.05 \text{ min}^{-1}$. The time course for the reaction with U1A was fit to the sum of two exponentials, yielding unwinding rate constants for the first phase of $k_{I}^{[RNP]} = 0.04 \pm 0.004 \text{ min}^{-1}$, and for the second phase of $k_{II}^{[RNP]} = (2.2 \pm 1.0) \times 10^{-4} \text{ min}^{-1}$. (D) Same as in C but with the RNA with a 3’ 25nt ssRNA region. The resulting time course without U1A yielded an unwinding rate constant of $k_r^{[RNA]} = 0.98 \pm 0.02 \text{ min}^{-1}$. The time course for the reaction with U1A yielded unwinding rate constants for the first phase of $k_{I}^{[RNP]} = 0.10 \pm 0.01 \text{ min}^{-1}$, and for the second phase of $k_{II}^{[RNP]} = (8.0 \pm 1.7) \times 10^{-4} \text{ min}^{-1}$. 
Having confirmed that Ded1p and ATP concentrations were saturating, it was important to verify that U1A did not inhibit Ded1p from unwinding RNA structures. Inhibition of duplex unwinding could conceivably prevent active U1A displacement by Ded1p. To examine this possibility, we created a tri-partite RNA substrate that consisted of a 20 nt 3’ ssRNA appended to a 13bp duplex adjacent to the U1A model duplex (Figure 2.5A). Without U1A, we expected that both the 13 bp duplex and the U1A model duplex would be unwound (Figure 2.5.A). However, if U1A did not inhibit Ded1p and was bound to the RNA, unwinding of the 13 bp duplex but not the duplex with the U1A binding site would be observed (Figure 2.5.A). Ded1p remodeled the 13 bp duplex regardless of the presence of U1A, suggesting that U1A did not prevent Ded1p from unwinding RNA structure (Figure 2.5.B). These data together with the results from the ATP and Ded1p titrations thus indicated that the inability of Ded1p to actively displace U1A from the RNA was an inherent feature of this DEAD-box protein.

We next examined whether the extent of RNA duplexes surrounding the U1A binding site prevented U1A displacement by Ded1p. As a DEAD-box protein, Ded1p is unable to translocate through a duplex (17), and it was thus possible that active U1A displacement by Ded1p could be seen in a substrate with less RNA secondary structure surrounding the U1A binding site. To this end we designed a substrate with a 4nt ssRNA region adjacent to the U1A binding site. Crystal structures of DEAD-box proteins bound to RNA suggest that 4 nt should be an adequate amount of RNA for Ded1p to bind (2). The 4 nt unpaired region was generated by substituting 4 nt in the substrate top strand with a poly-glycolic linker (Figure 2.6.A).
Figure 2.4. Saturating ATP and Ded1p concentrations for U1A displacement. (A) ATP titration of Ded1p U1A RNP remodeling. U1A model duplex with 3’ 25nt ssRNA was incubated U1A for 10min. and then Ded1p for 5min. The reaction was started with various concentrations of ATP and strand separation monitored by a non-denaturing PAGE. The amplitude of the first phase for biphasic kinetics was plotted against ATP concentration. The data was fit against a binding isotherm yielding $K_{1/2} = 1.4 \pm 1.0$. (B) Ded1p titration of Ded1p U1A RNP remodeling. Essentially the same reaction conditions as in A with the exception Ded1p concentration was varied. The data was fit against a binding isotherm yielding $K_{1/2} = 101 \pm 5$, $n = 5.0 \pm 2.3$. 
**Figure 2.5.** U1A does not inhibit Ded1p unwinding. (A) Scheme depicting unwinding of a tripartite RNA substrate containing a 13bp duplex and a model U1A RNA duplex. In the absence of U1A, both duplexes are unwound. In the presence of U1A, only the 13bp duplex is unwound. (B) Representative gel for unwinding of tripartite RNA complex from A. Mobilities of the RNA complex and the single-stranded RNA are indicated by cartoons on the left. Reactions were allowed to proceed for 5 min.
This substrate design also decreased the size of the remaining duplex from 19 to 15 bp. Remodeling of the poly-glycolic linker substrate was then compared to the reaction with a 19 bp RNA duplex. In addition, we monitored if appending a 3’ 25nt ssRNA region on the duplex affected strand separation. In the absence of U1A, Ded1p efficiently remodeled the linker substrates, with or without a 25nt 3’ ssRNA (Figure 2.6.B, 2.6.C upper panels). Substrates with complete RNA duplexes were remodeled less efficiently. Most likely, the additional 4 bp increases the duplex stability, and it is known that for DEAD-box proteins the unwinding rate constant decreases with duplex stability (Figure 2.6.B, 2.6.C lower panels). With U1A bound, a marked decrease in unwinding was observed with linker substrates. Almost no strand separation was observed with the complete RNA duplexes (Figure 2.6.B and 2.6.C).

We next examined the kinetics of RNA strand separation for the substrates with and without poly-glycolic linkers. Without U1A, Ded1p unwound the linker substrates without and with the 3’ 25nt ssRNA with apparent first order rate constants of $k_{unw} = 0.92 \pm 0.03 \text{ min}^{-1}$ and $k_{unw} = 0.91 \pm 0.01 \text{ min}^{-1}$ respectively. The complete RNA duplex substrates without and with the 3’ 25nt ssRNA were unwound with apparent first order rate constants of $k_{unw} = 0.17 \pm 0.01 \text{ min}^{-1}$ and $k_{unw} = 0.10 \pm 0.02 \text{ min}^{-1}$ respectively (Figure 2.6.D, 2.6.E). With U1A, however, very little to no strand separation was observed on the complete RNA duplexes, whereas the linker substrates followed biphasic timecourses similar to those seen with previous U1A substrates (Figure 2.6.D, 2.6.E cf Figure 2.3.C, 2.3.D). Interestingly, the Ded1p remodeling kinetics did not exactly mirror the spontaneous U1A dissociation kinetics (Figure 2.7).
Figure 2.6

A

G
U U
U A
A C A

5’-UUAGUACGUCCCA

U A

GC CCAG GUCUGUACGG

3’-AAUCAUGCGAGGGUCUGUCG

GGUC CAGACAUGCC

C C A
A U
C U
C

B

Ded1p  +  +  +  -
ATP    -  +  +  -
U1A    -  -  +  -
95°C    -  -  -  +

C

Ded1p  +  +  +  -
ATP    -  +  +  -
U1A    -  -  +  -
95°C    -  -  -  +
Figure 2.6. In the presence of U1A, Ded1p remodels the linker (gray) but not the RNA (black) substrates. (A) Design of RNP with poly-glycolic linker. Sequence of the RNA strands. Ded1p remodeling of RNAs without (B) or with (C) a 3’ 25nt ssRNA region (representative PAGE). Mobilities of the RNA complex and the single-stranded RNA are indicated by cartoons. (D) Time course of Ded1p unwinding of the linker (gray) or complete (black) RNA without a 3’ 25nt ssRNA with and without U1A bound. RNA strand separation by Ded1p with U1A bound (open circles) and in the absence of U1A (filled circles). The resulting time course for the reaction without U1A was fit against the integrated rate law for a first-order process, yielding $k_r^{[RNA, gray]} = 1.8 \pm 0.2 \text{ min}^{-1}$ and $k_r^{[RNA, black]} = 0.17 \pm 0.03 \text{ min}^{-1}$. The time course for the reaction with U1A was fit to the sum of two exponentials, yielding first phase of $k_r^{[RNP, gray]} = 1.4 \pm 0.4 \text{ min}^{-1}$ and $k_r^{[RNP, black]} = 1.5 \pm 0.8 \text{ min}^{-1}$, and for the second phase of $k_r^{[RNP, gray]} = (3.0 \pm 1.7) \times 10^{-3} \text{ min}^{-1}$ and $k_r^{[RNP, black]} = (5.8 \pm 8.9) \times 10^{-5} \text{ min}^{-1}$. (E) Same as in C but with the RNA with a 3’ 25nt ssRNA region. The resulting time course without U1A yielded an unwinding rate constant of $k_r^{[RNA, gray]} = >10 \text{ min}^{-1}$ and $k_r^{[RNA, black]} = 0.10 \pm 0.01 \text{ min}^{-1}$. The time course for the reaction with U1A yielded unwinding rate constants for the first phase of $k_r^{[RNP, gray]} = 3.0 \pm 0.8 \text{ min}^{-1}$ and $k_r^{[RNP, black]} = 0.11 \pm 0.03 \text{ min}^{-1}$; for the second phase of $k_r^{[RNP, gray]} = (6.3 \pm 2.1) \times 10^{-3} \text{ min}^{-1}$ and $k_r^{[RNP, black]} = (5.4 \pm 3.3) \times 10^{-4} \text{ min}^{-1}$.
Ded1p increased the dissociation of U1A in the first phase by ~2 fold as well as increased the amount of U1A displaced in the first phase by ~3 fold (Figure 2.7). The increase in the U1A dissociation rate constant suggests that there might be an active component in the displacement of U1A from the linker substrates. Thus, providing a single stranded region in the immediate vicinity of the U1A binding site enabled Ded1p to displace U1A from the RNA to a small, yet significant extent.

2.2.3: DISCRIMINATORY DISASSEMBLY OF RNP COMPLEXES BY Ded1p

Notwithstanding the results above, Ded1p was unable to actively displace U1A when its binding site was completely embedded in RNA secondary structure. The dissociation rate constant of U1A determined the rate constant by which Ded1p could disrupt these model RNPs. It occurred to us that this inability of Ded1p to actively displace U1A from these RNAs might, provide a straightforward means to enable a non-sequence specific DEAD-box protein to remodel similar RNA substrates in a discriminatory fashion. Because the U1A dissociation rate constant dictated the velocity by which Ded1p could disassemble the RNA strands, an RNA with a slight alteration in the U1A binding site that affected the U1A dissociation rate constant should be remodeled by Ded1p at a rate determined by this altered U1A dissociation rate constant. We therefore hypothesized that confronting Ded1p with a pool of similar RNAs containing slightly different U1A binding regions should result in a discriminatory remodeling of these RNAs in the presence, but not in the absence, of U1A (Figure 2.8.A)
**FIGURE 2.7.** Ded1p displaces U1A from substrates containing a poly-glycolic linker with a possible active component. Ded1p dependent active displacement (filled circles) or spontaneous dissociation of U1A from a RNA containing a poly-glycolic linker with a 25nt ssRNA appended to the 3’ of the duplex. The representative time courses were fit to the sum of two exponentials (dissociation rate constants were, for the first phase: $k^{I}_{RNP\text{filled}} = 3.0 \pm 0.8 \text{ min}^{-1}$, and for the second phase: $k^{II}_{RNP\text{filled}} = (6.3 \pm 2.1) \times 10^{-3} \text{ min}^{-1}$); and $k^{I}_{d\text{open}} = 1.2 \pm 0.8 \text{ min}^{-1}$, and for the second phase: $k^{II}_{d\text{open}} = (6.4 \pm 1.7) \times 10^{-3} \text{ min}^{-1}$). [Spontaneous U1A dissociation was measured by M.E. Fairman.]
To test this hypothesis, we designed a RNA with a slightly altered U1A binding site (Figure 2.8.B). We deleted three nucleotides from the U1A cognate site; otherwise the RNA was identical to the RNA duplex used above (Figure 2.1). When U1A bound to this altered RNA without and with a 3’ 25nt ssRNA, the U1A-RNA complex migrated in a non-denaturing PAGE with altered mobility (Figure 2.9.A, cf 2.2.A) and with an apparent affinity of $K_D = 1.9 \pm 0.2$ nM and $K_D = 13.5 \pm 0.7$ nM respectively (Figure 2.9.B), i.e., only slightly weaker than the RNA with the authentic U1A binding site (cf. Figure 2.2.B). However, U1A dissociated from the altered RNAs significantly faster than from the wtRNA (Figure 2.9.C, cf 2.2.C). As observed for RNAs containing the wild-type U1A binding site, U1A dissociation followed a biphasic time course whose shape did not change upon alterations in the reaction conditions and increases in the U1A concentration (data not shown). Thus, as seen with the wt substrate, the biphasic U1A dissociation kinetics from the altered RNA most likely reflects inherent heterogeneity or an induced-fit binding mode of the U1A–RNA complex (91, 92).

To further characterize differences between the two U1A substrates, we measured the stability of the duplexes and the U1A footprint on the different RNAs. To measure the stability for the wildtype and altered substrates, each radioactively labeled duplex was incubated at various temperatures for 5 minutes, immediately placed on ice, and loaded onto a non-denaturing PAGE. The melting curves for both substrates do not significantly differ and there is only a difference of $\sim 2^\circ$C between $T_m$’s (Figure 2.10.A). These data indicate that both substrates have similar stabilities.
Figure 2.8. Substrate design of the altered U1A model RNP. (A) Scheme depicting Ded1p unwinding of U1A model RNA duplexes with different off-rate constants. Without U1A (i), both RNA substrates are unwound indiscriminately. In the presence of U1A (ii), U1A dissociates faster from the gray duplex than from the black duplex allowing Ded1p to remodel only the gray duplex. (B) Altered U1A RNP design. Three nucleotides were deleted from the upper RNA strand (indicated by red oval) of the RNA complex with the authentic U1A binding site (Figure 2.1).
Figure 2.9

A

B

C

- +

0.1 1 10 100 1000

U1A [nM]

0 0.2 0.4 0.6 0.8 1

Fraction Bound

0 40 80 120 160

Time [min]

Fraction Free
FIGURE 2.9. Altered U1A based RNP. (A) U1A binding to the substrate without a 3’ 25nt ssRNA. Radiolabeled RNA complex (0.5 nM) was incubated without (left lane) and with 20 nM U1A (right lane) under reaction conditions for 10 minutes and applied to 8% non-denaturing PAGE. The cartoons indicate free and U1A (oval) bound RNA complexes. (B) Equilibrium binding of U1A to the RNA duplex without (filled circle) and with (open circle) a 3’ 25nt ssRNA. Data points represent the average of at least three independent measurements. Error bars represent one standard deviation. Data were fit to the Hill-equation ($K_D^{\text{filled}} = 1.9 \pm 0.2$ nM, $n = 1.4 \pm 0.2$; $K_D^{\text{open}} = 13.5 \pm 0.7$ nM, $n = 1.7 \pm 0.1$). (C) Spontaneous dissociation of U1A from the RNA without (filled circles) and with (open circles) a 3’ 25nt ssRNA. The representative time courses were fit to the sum of two exponentials (dissociation rate constants were, for the first phase: $k_d^{\text{filled}} = 0.13 \pm 0.1$ min$^{-1}$, and for the second phase: $k_d^{\text{filled}} = (1.3 \pm 0.1) \times 10^{-2}$ min$^{-1}$); and $k_d^{\text{open}} = 2.2 \pm 0.6$ min$^{-1}$, and for the second phase: $k_d^{\text{open}} = (5.3 \pm 0.7) \times 10^{-3}$ min$^{-1}$).
To determine the U1A footprint on the wildtype and altered RNAs, we performed micrococcal nuclease protection assays. Each duplex was incubated with or without U1A, digested by micrococcal nuclease, and loaded onto a denaturing PAGE. Strand A for the wildtype and altered substrate (Figure 2.11.A) showed protection patterns for several bases around the loop that binds U1A (Figure 2.11.B). There was a slight difference in the number of bases protected. U1A protected the ninth adenine from the 5’ end (A₉) of the wildtype strand A whereas the altered substrate did not. Despite the one base difference between the wildtype and altered substrates, the remaining protection patterns were identical. We concluded that U1A binds strand A of the wildtype and altered substrates through very similar contacts.

For strands B and C of the wildtype and altered RNAs respectively, increased protection was observed for bases U₂₁-G₂₄ of the altered RNA in strand C when compared to strand B of the wildtype RNA (Figure 2.11.A, compare to wildtype bases U₂₄-G₂₇ in strand B, Figure 2.11.C). The increased protection of U₂₁-G₂₄ on strand C most likely is due to a U1A induced fold that prevents nuclease digestion at positions U₂₁-G₂₄. Consistent with this notion, the altered substrate is theoretically capable of assuming two equally stable RNA conformations (data not shown). Most importantly however, the pattern of nuclease digestion within the loops of strands B and C did not differ significantly (Figure 2.11.C). Therefore, U1A apparently binds the loop in strand C in the same manner as the loop in strand B despite the removal of 2 bases. Collectively, the footprint results on
Figure 2.10. Characterization of altered U1A RNP. Melting curves for wildtype (filled circles) and altered (open circles) U1A model duplexes without 3’ 25nt ssRNA. Labeled duplex was incubated at various temperatures for 5 minutes, immediately placed on ice, and loaded onto a non-denaturing PAGE. Experiments were conducted twice and averaged. Error bars represent the difference between the data. Data was fit with a trend line.
each strand of both substrates (Figure 2.11.A, ovals), indicate that U1A binds the loops of both duplexes in a similar fashion.

Equal wildtype and altered duplex stabilities confirmed that we were monitoring differences in U1A displacement and not differences in strand separation. Determining the U1A footprint for both RNA duplexes demonstrated that the altered substrate’s decrease in affinity and increase in the dissociation rate constant for U1A was not the result of a change in U1A binding. The characterization of the substrates revealed that the removal of the nucleotides in the altered substrate predominately affected the dissociation rate constant and the apparent equilibrium binding of U1A. Neither duplex stability nor the mode of U1A binding with respect to the footprint was changed significantly. The difference between the dissociation kinetics of the wildtype and altered RNAs thus rendered the altered RNA suitable for determining whether Ded1p would remodel the RNAs differently if U1A was bound.

To test whether U1A would enable Ded1p to unwind both RNAs in a discriminatory fashion, we combined both RNA complexes in an equimolar ratio and monitored strand separation by Ded1p with and without U1A (Figure 2.12.A). Without U1A, all substrates were readily unwound by Ded1p to a virtually identical degree (Figure 2.12.A). With U1A, Ded1p unwound both RNAs in a clearly differential fashion. The RNA with the altered U1A binding site was unwound to a significantly greater extent than the RNA with the authentic U1A binding site (Fig 2.12.A). The same observations were made when the substrates contained a 3’ 25nt ssRNA (Figure 2.12.B).
Figure 2.11

A

WT

G
U
U
A
A
A

B: 5’-CCCAGACAGC CCAG GUCGUACGG-3’
A: 3’-GGGUCUGUCG GGUC CAGACAUGCC-5’

Alt

G
U
U
U
A

C: 5’-CCCAGACAGC CCAG GUCGUACGG-3’
A: 3’-GGGUCUGUCG GGUC CAGACAUGCC-5’

B

Marker Ladder
- + + U1A

32nt-

U12

16nt-

Loop

Strand A
Figure 2.11. Nuclease protection assay. (A) Wildtype (WT) and altered (Alt) U1A model duplexes used for protection assay. Strands are labeled either A for bottom strand, and B or C for wt or alt top strands respectively. Yellow ovals represent bases protected by the addition of U1A. (B) Nuclease digestion of wt and alt RNA duplexes with strand A radioactively labeled with or without U1A. (C) Nuclease digestion of wt labeled strand B and alt labeled strand C with or without U1A.
An identical experiment with NPH-II showed no comparable differences in the unwinding of both RNAs in the presence or absence of U1A, thereby verifying the integrity of the RNAs and the RNPs (93).

Kinetic analysis of the unwinding time courses with Ded1p revealed that the disassembly of wildtype and altered RNA complexes with or without a 3’ 25nt ssRNA with bound U1A mirrored the dissociation kinetics of U1A from the respective RNA (Figure 2.12.C, 2.12.D). Thus, Ded1p did not actively displace U1A from either RNA. In the absence of U1A, however, Ded1p unwound both wildtype and altered RNAs with virtually identical rate constants (Figure 2.12.C, 2.12.D). These results demonstrate that U1A binding to distinct binding sites in otherwise similar RNAs enables Ded1p to differentially remodel these RNAs. NPH-II, which actively dislodges U1A from wildtype and altered complexes with 3’ 25nt ssRNA regions, is unable to differentiate between both RNAs, either with or without U1A bound (93).

2.2.4: REMODELING OF A ssRNA BINDING PROTEIN BY Ded1p

The RNA secondary structure surrounding the U1A binding site prevented Ded1p from actively remodeling U1A. However, when a break in the secondary structure adjacent to the U1A binding site was inserted, Ded1p displaced the U1A with some active component. These observations suggested that RNP remodeling might be linked to the ability of the enzyme to translocate. Since Ded1p as a DEAD-box protein does not translocate, it cannot unwind the RNA duplex and remove the protein before falling off
Figure 2.12

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C

Fraction Product vs. Time [min]

D

Fraction Product vs. Time [min]
Figure 2.12. Ded1p disassembles the two RNA complexes without (A) or with (B) a 3’ 25nt ssRNA in a discriminatory fashion. Ded1p disassembles the altered RNA complex more efficiently than the complex with the authentic U1A binding site when U1A is bound but not without U1A. Mobilities of the RNA complexes and the single-stranded RNAs are indicated by cartoons. The altered RNA is in gray, the RNA with the authentic U1A binding site in black. (C) Representative time courses of DED1-catalyzed unwinding of wt (black) and altered (grey) RNPs without (filled circles) and with (open circles) U1A. Strand separation of the altered RNA complex without U1A was fit to a single exponential, yielding $k_r^{[RNA]} = 1.2 \pm 0.09$ min$^{-1}$. Strand separation of the altered RNA complex with U1A present was fit to a sum of two exponentials yielding for the first phase: $k_r^{[RNP]} = 0.043 \pm 0.005$ min$^{-1}$, and for the second phase: $k_2^{[RNP]} = (3.3 \pm 0.7) \times 10^{-3}$ min$^{-1}$. (D) Same as in C but duplexes contained a 3’ 25nt ssRNA. Strand separation of the altered RNA complex without U1A was fit to a single exponential, yielding $k_r^{[RNA]} = 1.63 \pm 0.15$ min$^{-1}$. Strand separation of the altered RNA complex with U1A present was fit to a sum of two exponentials yielding for the first phase: $k_1^{[RNP]} = 0.33 \pm 0.04$ min$^{-1}$, and for the second phase: $k_2^{[RNP]} = (4.8 \pm 0.7) \times 10^{-3}$ min$^{-1}$. Kinetic data for strand separation of the RNA complexes with the authentic U1A binding site (with and without U1A) are reported in Figure 2.3.
the RNA. However, it was unclear whether Ded1p was able to displace proteins that bind to completely single stranded RNA.

To test this possibility, we chose a model system in which RNA secondary structure plays no role in protein binding. The complex is formed between the tryptophan RNA-binding attenuation protein (TRAP) (Figure 2.13.A) and its specific 53 nucleotide cognate RNA (Figure 2.13.B). No RNA secondary structure surrounds or is contained within the TRAP-binding site (94, 95). TRAP binds to its cognate RNA in a sequence-specific manner as an 11-unit oligomer. Its affinity can be modulated by tryptophan; that is, increasing tryptophan concentrations stabilize the RNA protein complex (94).

Two RNAs containing the TRAP binding site were prepared. One was composed only of the minimal 53-nucleotide TRAP-binding region, whereas the other had a single-stranded, 3’-terminal, 24nt extension in order to provide a binding site for Ded1p (Figure 2.13B). Both RNAs formed distinct complexes with TRAP that were visualized by non-denaturing PAGE (Figure 2.14.A). TRAP bound to both RNAs with subnanomolar affinity and dissociated in a biphasic fashion (Figure 2.14.B). The TRAP-RNA complex containing a 3’ ssRNA was actively remodeled by NPH-II (96).

We first assessed the ability of Ded1p to disrupt the TRAP-RNA complexes. TRAP-RNA complexes were formed prior to the reaction for 10 min, Ded1p was then added, and incubation was continued for at least 5 min. Remodeling reactions were started by adding a mixture of ATP and RNA scavenger. The RNA scavenger consists of DNA
**FIGURE 2.13.** Structure of the TRAP protein and substrate design. (A) Structure of the TRAP protein as determined by x-ray crystallography. (B) RNP design. Sequence of the RNA strands. TRAP binds to the penta-nucleotide repeat as depicted by gray ovals.

Figure 2.14

A

Figure 2.14. Spontaneous dissociation of TRAP protein. (A) Representative time course for TRAP dissociation from either RNA with (upper panel) or without (lower panel) 3’ ssRNA. Reactions were started by adding DNA oligonucleotides that bind the RNA and prevent TRAP rebinding. Aliquots were removed over 30 minutes. TRAP-bound RNA and free TRAP substrate RNA are indicated by the cartoons on the left. (B) Quantification of spontaneous dissociation of TRAP from RNA with (filled circles) or without (open circles) 3’ ssRNA.
oligonucleotides that hybridize to the TRAP-binding site and thus prevent rebinding of TRAP once it has been displaced. Ded1p does not bind to DNA with high affinity and therefore was not sequestered by the RNA scavenger (18). Displacement reactions were monitored by non-denaturing PAGE (Figure 2.15.A, 2.15.C). Ded1p could not accelerate TRAP dissociation from either RNA under any condition tested (Figure 2.15.B, 2.15.D). The failure to actively displace TRAP was not due to compromised Ded1p enzyme under the reaction conditions because Ded1p unwound RNA duplexes as expected in control reactions (Figure 2.15.A, 2.15.C). Collectively, these data showed that Ded1p was not able to actively dissociate TRAP from its cognate RNA despite the lack of RNA secondary structure.

2.3 DISCUSSION

In this study, we have shown that Ded1p can not displace U1A and TRAP from their cognate RNAs in a clearly active fashion. Yet, we have also shown that the “inability” to actively displace proteins from RNA can provide the non-sequence specific Ded1p with a means to nonetheless disassemble very similar RNA complexes in a discriminatory fashion.

While previous observations have demonstrated that NPH-II can actively displace U1A and TRAP from their cognate RNA binding sites (40, 96), we have shown here that Ded1p can not. These studies highlight the differences between DExH proteins and DEAD-box proteins. DExH proteins like NPH-II have a high rate of ATP hydrolysis that fuels their processive unidirectional translocation on RNA (19, 97).
Figure 2.15
Figure 2.15. Ded1p does not actively remodel TRAP-RNA complexes. (A) Representative time course for TRAP-RNP remodeling by Ded1p. Reactions with the RNA containing the ssRNA were performed in the presence of a duplex control substrate (16 base pairs containing a 24nt ssRNA 3’ to the duplex region). Aliquots were removed over 30 minutes. TRAP-bound RNA, free TRAP substrate RNA, control duplex substrate, and unwound control substrate are indicated by the cartoons on the left. (B) same as A but with RNA not containing 3’ ssRNA. (C) Quantification of Ded1p assisted dissociation (filled circles) and spontaneous dissociation (open circles) of TRAP from RNA substrate with RNA containing ssRNA 3’ the TRAP binding site. (D) Same as in C but with RNA not containing 3’ ssRNA.
The physiochemical properties of NPH-II enable it to actively displace proteins from RNA by disrupting RNA-protein interactions as it translocates along the RNA. In contrast, Ded1p does not hydrolyze ATP at the same high velocity that NPH-II does (65). Furthermore, Ded1p, as a DEAD-box protein, does not translocate along RNA (17). Instead, Ded1p most likely binds RNA in an ATP-dependent fashion, hydrolyzes the ATP, and then dissociates. These physiochemical properties of Ded1p and other DEAD-box proteins apparently limit the ability of these enzymes to disrupt RNA-protein interactions.

Our results demonstrating U1A displacement from the RNA duplexes containing a polyglycol linker indicated that the context of a RNP might also determine whether a DEAD-box protein is able to actively displace proteins from RNA. Consistent with this notion, active displacement of the EJC and the U1snRNP from RNA by Ded1p had also been observed (93, 96). The EJC is a multi-protein complex that binds RNA with a footprint of approximately 8-10 nt (96). Ded1p binding to the RNA may disrupt enough of those 8-10 nt RNA-protein interactions to actively displace the EJC from the RNA (96). In contrast, Ded1p can not disrupt enough RNA-protein contacts between the TRAP protein and its 53 nt binding site. These data suggest that proteins with a small binding site maybe actively displaced by Ded1p. The U1snRNP is a RNP that binds its cognate RNA binding site through a combination of a 7 bp helix and RNA-protein interactions (93). While it is not known exactly how Ded1p actively displaces the U1snRNP from its RNA binding site, it is most likely through disruption of the 7 bp helix between the U1snRNP and the RNA. U1snRNP displacement through disruption of the multiple RNA-protein
interactions is unlikely because Ded1p would have to disrupt a potential 30 nt of RNA-protein interactions (93, 98). To reconcile the observations made during the remodeling of the above RNPs by Ded1p it is useful to propose a basic mechanism by which DEAD-box proteins displace proteins from RNA (Figure 2.16).

We propose that the ability of a DEAD-box protein to actively disrupt a given RNP is based on the capacity of the enzyme to capture nucleotides that are normally part of the RNA–protein interface of the RNP. This reduction in the number of RNA–protein contacts increases the propensity of the protein(s) to dissociate from the RNA at a rate greater than that of spontaneous RNP dissociation, i.e. the protein is actively displaced from the RNA (Figure 2.16.A). This mechanism would be consistent with Ded1p’s ability to displace multi-component RNPs with small binding sites such as the EJC and U1snRNP. However for homo-oligomeric RNPs that have much larger binding sties, DEAD-box proteins dissociate from the RNA before capturing the critical number of nucleotides necessary to accelerate dissociation of the RNP, i.e. no active protein displacement is observed (Figure 2.16.B).

It is unclear how exactly the nucleotides are captured by the DEAD-box proteins. Conceivably, the DEAD-box helicase could exert force on the other protein to ‘free’ one or several nucleotides, although it is not known whether DEAD-box proteins can produce sufficient force when binding RNA. Alternatively, the DEAD-box helicase could simply sequester transiently fraying nucleotides on the RNA. We note that binding of the
Figure 2.16. Possible mechanism for protein displacement by DEAD-box proteins. (A) Schematic representation for the displacement of a protein with a small RNA binding site. The line represents the RNA. (B) Schematic representation of a DEAD-box protein unable to dissociate a protein with a large RNA binding site.
helicase on single-stranded nucleic acid and capture of fraying nucleotides may also be important for the unwinding of RNA duplexes by DEAD-box protein (17). There, a helicase is proposed to capture fraying nucleotides from anywhere within the helix (17). It is perhaps not surprising that similar mechanisms may underlie both duplex unwinding and protein displacement by DEAD-box proteins enzymes.

Because of the limited amount of data for RNP remodeling by DEAD-box proteins, other mechanisms by which these enzymes cause active protein displacement should not be discounted. For example, it may be possible that, instead of capturing one or few nucleotides at a time, DEAD-box proteins may simply force the entire protein off the nucleic acid by physical clashes between protein domains. While it is unknown whether DEAD-box proteins can exert sufficient force when binding the RNA, as mentioned above, it is well established that DEAD-box proteins change their domain orientations upon ATP binding/hydrolysis (99). Such large scale conformational changes could be used to induce a physical clash between the DEAD-box enzyme and other proteins.

The discussion above illuminated possible mechanisms by which DEAD-box enzymes actively displace other proteins from RNA, but the inability of DEAD-box proteins to actively dislodge other proteins might be equally significant, most notably for enabling the non-sequence specific DEAD-box proteins to act in a discriminatory fashion. We underscore that the mechanisms for the discriminatory function of Ded1p discussed below are based solely on the data collected with artificial model systems in vitro. We do not imply that Ded1p targets either of the tested model substrates, U1A or TRAP in vivo.
We further do not suggest that Ded1p invariably functions in its physiological environment as described in this model study. Finally, we note that the mechanism for discriminatory RNP remodeling does in no way preclude the recruitment and perhaps specific activation of DEAD-box proteins by their specific targets. Rather, the proposed discriminatory function of DEAD-box proteins may complement their recruitment to specific targets by (i) preventing disassembly of RNPs beyond intended target regions, (ii) allowing the timing of RNA remodeling reactions without directly affecting the DEAD-box protein, and (iii) providing one possible way to consider conformational proofreading by RNA helicases in straightforward terms (see discussion below).

The inability of Ded1p to actively displace certain proteins from RNA highlights the possibility for discriminatory RNP remodeling on two levels. The first level of discrimination is based on the ability of Ded1p to actively (efficiently) disassemble only certain RNPs. Possible mechanisms for this phenomenon have been discussed above. Thus, confronting an enzyme like Ded1p with a pool of different RNPs (with small or large RNA-protein interaction sites) will result in the remodeling of some but not other RNPs. It is unclear to which degree this situation resembles physiological reactions. Nonetheless, this level of discriminatory RNP remodeling is, despite its simplicity, not a trivial finding. This is (i) since not all RNA helicases behave in a similar fashion and (ii) because no direct interactions between the DEAD-box protein and other co-factors and no modifications of the biochemical activities of the enzymes are involved.
The second level of discrimination is less obvious than the one discussed above. We have shown here that Ded1p was not only unable to actively displace U1A from its cognate RNA, but also that the dissociation rate constant of U1A determined how fast Ded1p remodeled RNAs with bound U1A. Alterations in the RNA that change the U1A off-rate affect the rate of RNA remodeling by Ded1p and thus enable the non-sequence specific Ded1p to discrimately remodel RNAs based on only slight sequence differences. By unwindng RNAs containing altered U1A binding sites at a faster rate than RNAs with an authentic U1A binding site, in principle, Ded1p could be viewed as “proofreading” for RNAs with an authentic U1A binding site (RNAs with altered U1A binding sites are preferentially disassembled, i.e., “discarded”). Thus, “proofreading” by RNA helicases could be considered in straightforward terms, although physiological ramifications of this possible “proofreading” mechanism remain unclear. However, we note that the proposed mode for “proofreading” is consistent with the ATP-dependent kinetic proofreading function of the spliceosomal DExH/D protein Prp16 (100), and with activity modulations of the DExH/D proteins Prp22 and Brr2 by the spliceosomal protein Prp8 (101, 102).

The inability of Ded1p to actively displace other proteins from RNA also illuminates a straightforward means to time RNA rearrangement steps by DEAD-box enzymes without the need to establish specific protein-protein interactions. Events unrelated to the DEAD-box protein function, such as a transesterification step during pre-mRNA splicing, or protein phosphorylation may simply alter the off-rate of a regulatory protein, and thereby time the ATP-driven remodeling function of a DEAD-box protein. It is not known
whether in a physiological context the timing of some DEAD-box protein-catalyzed RNA rearrangements occur according to this mechanism. However, it may be attractive to specifically test whether certain genetic interactions between DEAD-box enzymes and RNA binding proteins arise due to such “control” of DEAD-box enzymes by RNA binding proteins.

We note that NPH-II, which actively displaces U1A from its cognate RNA, could not be “controlled” by U1A. Consequently, NPH-II could not preferentially remodel any of the RNA complexes tested. Therefore, it may be critical for possible discriminatory functions of DExH/D proteins that the enzymes in question are unable to actively displace certain other proteins from RNA. Consequently, DExH/D proteins such as Ded1p may have mechanistic characteristics that result in less potent RNA helicase or RNPase activities in vitro. On the other hand, viral proteins such as NPH-II might have evolved as “cleaners” that indiscriminately remove other proteins and RNAs from a target RNA.
CHAPTER 3: **Ded1p FACILITATES RNA BINDING BY THE TRANSLATION INITIATION FACTOR 4G**

### 3.1: INTRODUCTION

The observed helicase activity of DEAD-box proteins results from the combination of their biochemical activities; i.e. RNA binding, hydrolysis of ATP, and strand separation. Helicase cofactors can positively or negatively affect any one of these biochemical activities (5, 44, 49). Modulation of DEAD-box proteins can lead to enhanced biochemical function (58), or prevent dissociation of the DEAD-box protein from RNA (5). However, not every protein that interacts with DEAD-box proteins affects the biochemical activities of DEAD-box proteins (59). Notwithstanding these observations, the effects of cofactors on DEAD-box proteins and DEAD-box proteins on cofactors are not well understood. To investigate this issue, we tested how Ded1p and one of its cofactors, eIF4G, influenced each others biochemical activities.

### 3.2: RESULTS

#### 3.2.1: **EXPRESSION OF A C-TERMINALLY HA-TAGGED Ded1p**

To identify proteins that interact with Ded1p *in vivo*, we replaced the genomically encoded Ded1p with a hemagglutinin (HA)-tagged Ded1p, performed immunoprecipitations with a monoclonal antibody (mAb) against the HA-tag, and analyzed the co-immunoprecipitated proteins by mass spectrometry. Replacement of the genomic DED1 with HA-tagged DED1 was performed by homologous recombination. A DED1 gene with a C-terminal 3x HA tag was created by PCR, and the selectable marker URA3 was inserted after the poly-adenylation signal in the 3’ untranslated region (UTR) of the
DED1 gene (Figure 3.1.A). The HA-tagged DED1 construct was then transformed into the BY4741 yeast strain and transformants were selected on plates lacking uracil.

To confirm correct recombination and the presence of the HA-tag, we performed PCR on the genomic DNA of the background strain (BY4741) and the HA-tagged DED1 transformed strain (BY4741.HA). With primers corresponding to the C-terminus of the DED1 gene, we amplified the DNA and analyzed the PCR products on an agarose gel (Figure 3.1.B, top panel). Amplification of the BY4741 DNA resulted in a band migrating at approximately 100bp, as expected (Figure 3.1.B, top panel). The band for the homologously recombined yeast migrated more slowly with a size indicative of the presence of a 3x HA tag (199bp). The lack of a band corresponding to the control (BY4741) size indicated the absence of a wildtype Ded1p in the BY4741.HA yeast strain and confirmed that homologous recombination correctly occurred.

We then verified the addition of the URA3 gene. PCR of the genomic DNA was performed with primers outside the boundaries of our HA-tagged DED1 construct (Figure 3.1.B, bottom panel). For the wildtype BY4741 strain, a band migrating at the expected size of 4kb was visible. For the BY4741.HA strain, a product migrating at approximately 5.0kb was seen, indicating the presence of the URA3 gene.

We subsequently examined expression of the HA tagged Ded1p protein. Yeast lysates were generated from BY4741 and BY4741.HA strains. Western blot analysis was performed with both lysates using a polyclonal antibody (pAb) α-Ded1p (Figure 3.1.C).
Figure 3.1. Creation of a C-terminally HA tagged Ded1p yeast strain (A). A DNA construct containing DED1 with a HA tag at its C-terminus and the selectable marker URA3 was transformed into the BY4741 strain and homologous recombination occurred. Positive clones containing the recombined gene were selected for on plates lacking uracil.

(B) PCR was used to confirm the homologous recombination. The primers used are depicted as arrows over the cartoon for each genome. (C) Western blots of yeast lysates probed with α-Ded1p, α-HA, and α-alpha Tubulin reveal the presence of a HA tagged Ded1p in the BY4741.HA strain that is expressed at a 1.0:1.5 ratio to BY4741.
Blots with the BY4741.HA lysate showed a band consistent with the addition of a HA tag on Ded1p. Probing with the monoclonal antibody (mAb) α-HA yielded no visible signal in the BY4741 lysate. Using the BY4741.HA lysate, the mAb α-HA confirmed the presence of a HA-tagged protein that migrated at the same size as the Ded1p from the pAb α-Ded1p blot. Together, these data indicated that HA tagged Ded1p was expressed in the BY4741.HA strain.

Next we compared the expression levels of WT and HA Ded1p. Using alpha tubulin as a loading control, we found that the HA-tagged Ded1p in the BY4741.HA strain was expressed at approximately 66% of the Ded1p level in the BY4741 strain. The reduced amount of Ded1p led to a slight increase in the doubling time of 108 minutes for WT to 120 minutes (~10% increase). These data indicated that Ded1p was not expressed at higher levels than WT, and we therefore judged the BY4741.HA strain acceptable for further experiments.

3.2.2: IDENTIFICATION OF POTENTIAL Ded1p COFACTORS

To identify proteins associated with Ded1p, we performed co-immunoprecipitation experiments. We immunoprecipitated Ded1p from BY4741.HA (HA) lysate using the mAb α-HA antibody and subsequently eluted Ded1p and bound proteins from the antibody by addition of HA peptide. The proteins from the HA peptide eluate were precipitated by TCA and separated by SDS PAGE (Figure 3.2.A). Several replications of this procedure produced consistent protein staining patterns. To identify the proteins that co-immunoprecipitated with Ded1p, mass spectrometry analysis was conducted with
proteins eluted from the gel. Most of the detected proteins are involved in either sugar metabolism or RNA processing, including ribosome biogenesis, RNA export, translation, and even RNA degradation (Figure 3.2.B). The variety of the co–immunoprecipitated proteins that are involved in RNA metabolism is consistent with the notion that Ded1p is involved in multiple RNA processing events (77).

We chose to focus further analysis on the interaction between Ded1p and eIF4G. Like Ded1p, eIF4G is involved in translation initiation (46), and had already been shown to interact with Ded1p through co-immunoprecipitation in a global proteomic survey (103). In addition, a lethal phenotype was observed for a knockout of TIF4631 (eIF4G) in the background of a temperature sensitive DED1 mutant indicating a genetic link between the two genes and thus suggesting an essential physiological role for the interaction between the two proteins (104).

3.2.3: EIF4G DIRECTLY BINDS Ded1p

To confirm the interaction between eIF4G and Ded1p, we performed western blot analysis on immunoprecipitates of Ded1p. To elucidate whether eIF4G and Ded1p interacted through RNA, we conducted the immunoprecipitations in the presence and absence of RNase A (Figure 3.3.A). eIF4G was only detected in the immunoprecipitate containing a HA tagged Ded1p, and addition of RNase A did not affect the interaction
Figure 3.2. Identification of Ded1p associated proteins through co-immunoprecipitation.
(A) Flow chart depicting immunoprecipitation procedure used to identify Ded1p cofactors. (B) Eluates from HA immunoprecipitations of BY4741 and BY4741.HA yeast lysates were loaded onto a 8% SDS-PAGE gel and coomassie stained. Bands were then cut out for LC-MS to identify the corresponding proteins. Proteins involved in mRNA processing, metabolism, or miscellaneous processes as well as proteins that have been shown to co-immunoprecipitate with Ded1p are indicated by a shaded box next to the gene name.
between eIF4G and Ded1p. Use of a RNAse cocktail mix in addition to RNAse A was also utilized and no difference in the above results was observed (data not shown). These data indicate that Ded1p and eIF4G do not associate through RNA. Rather, eIF4G and Ded1p interact in a RNA independent manner either through other proteins or direct protein-protein contacts.

To distinguish between these two possibilities, we performed GST-pulldowns with purified proteins. Ded1p was incubated with GST or GST-eIF4G. BSA was added to prevent non-specific interactions. After washes, samples were analyzed by western blot analysis (Figure 3.3.B). Since Ded1p and BSA migrated at approximately the same size on the SDS-PAGE, the Ded1p input bands were distorted by the BSA (Figure 3.3B, lanes 1-3). The GST-pulldowns performed with GST or beads alone failed to pull down Ded1p (Figure 3.3.B, lanes 4 and 6). GST-eIF4G pulled down Ded1p, indicating that eIF4G directly associated with Ded1p (Figure 3.3B, lane 5). Taken together, immunoprecipitations and pulldown experiments showed that Ded1p interacted with eIF4G via direct protein-protein interactions.

We next explored whether the presence of ATP or ATP analogs influenced the interaction of Ded1p with eIF4G. To investigate the effect of nucleotides on the interaction between GST-eIF4G and Ded1p, GST-pulldowns were performed in the presence of several ATP analogs. As before, the presence of BSA distorted the Ded1p input lanes. GST-pulldowns in the presence of ADPNP, ATP, or ADP-AlF₆ showed no changes (data not shown). Interestingly, ADP slightly inhibited the association between
**Figure 3.3.** eIF4G directly binds Ded1. (A) Yeast lysates were incubated with mAb α-HA beads to isolate HA tagged Ded1. After washing, the beads were heat denatured along with the inputs and loaded onto 8% SDS page gels and probed with pAb α-eIF4G or pAb α-Ded1p. (B) Glutathione sepharose beads were incubated with either purified Gst and Ded1p (lanes 1 & 3), GST-eIF4G and Ded1p (lanes 2 & 5), or Ded1p only (lanes 3 & 6). After 3 washes, samples were loaded onto a 6-10.5% SDS-PAGE gel and probed with α-eIF4G, α-Ded1p, or α-S Tag (for GST).
Ded1p and GST-eIF4G compared to the reaction without ATP analogs. However, ADP-BeF<sub>x</sub>, but not BeF<sub>x</sub> alone, greatly increased the stability of the Ded1p interacting with GST-eIF4G (Figure 3.4). Since DEAD-box protein structure is influenced by the presence of nucleotides (2), the modulation of Ded1p association to eIF4G by nucleotides suggested that the conformation of Ded1p regulates the interaction between Ded1p and eIF4G.

### 3.2.4: PURIFIED EIF4G/4E BINDS RNA

To examine the functional consequences of eIF4G binding to Ded1p, we generated purified eIF4G. Because eIF4G has been shown to be stable only when complexed with the cap binding protein eIF4E, we purified a eIF4G/4E complex (105). The presence of eIF4E does not further affect the interaction between eIF4G and Ded1p as shown below; moreover genetic studies (104) indicate that eIF4E does not interact with Ded1p.

A N-terminally GST-tagged and C-terminally His-tagged eIF4G was co-expressed in *E. coli* with the cap binding protein eIF4E. After passes over Ni-NTA, ion exchange, and glutathione-sepharose columns, eIF4G and eIF4E were purified in a 1:1 molar ratio complex. Thrombin cleavage from the glutathione-sepharose resulted in the release of full-length eIF4G with a N-terminal S-tag and a C-terminal His-tag complexed to eIF4E (Figure 3.5.A).

To ensure that the purified eIF4G protein was functional, we examined the RNA binding activity of eIF4G/4E. We incubated a radioactively-labeled 71 nucleotide (nt) single
Figure 3.4. ATP analogs affect the Ded1p/eIF4G interaction. Glutathione sepharose beads were incubated with GST-eIF4G, Ded1p, and either no nucleotide, ADP, or ADP-BeFx. After 3 washes, samples were loaded onto a 8% SDS-PAGE gel and probed with α-S Tag (for GST-eIF4G), or α-Ded1p.
stranded (ss) RNA with increasing concentrations of eIF4G/4E and loaded the reactions onto a non-denaturing PAGE (Figure 3.5.B). eIF4G/4E bound the 71 nt RNA with an apparent dissociation constant of $K_D = 123 \pm 23$ nM (Figure 3.5.C), indicating that the eIF4G is functional with respect to RNA binding.

We next probed the minimal RNA size required for efficient eIF4G/4E binding by a binding competition assay. eIF4G/4E was incubated with a radioactively labeled 71nt ssRNA and unlabeled ssRNAs ranging in size from 16 – 66 nt, and eIF4G/4E binding to the 71nt ssRNA was monitored by non-denaturing PAGE. Once the unlabeled ssRNA reached an adequate length to compete for binding, eIF4G/4E binding to the labeled 71 nt ssRNA was expected to be reduced (Figure 3.6.A). We observed that ssRNAs shorter than 21 nt did not compete for eIF4G/4E binding, whereas ssRNAs longer than 35 nt prevented binding of eIF4G/4E to the 71 nt ssRNA (Figure 3.6.B). These data indicate that for efficient eIF4G/4E binding, the minimal ssRNA size must be $\geq 35$ nt.

### 3.2.5: EIF4G DOES NOT MODULATE Ded1p’s RNA-STIMULATED ATPASE ACTIVITY IN VITRO

Having verified the Ded1p/eIF4G interaction and that purified eIF4G was functional, we probed whether eIF4G modulated the activities of Ded1p. To examine eIF4G’s effect on the RNA stimulated ATPase activity of Ded1p, we monitored the rate at which Ded1p hydrolyzed ATP in the absence or presence of eIF4G (Figure 3.7). Ded1p’s ATPase activity was measured using a coupled ATP/NADH system, where ATP hydrolysis is coupled to NADH oxidation through the enzymes pyruvate kinase (PK), lactate
Figure 3.5. Purified eIF4G binds RNA. (A) Flow chart depicting purification of full-length GST-eIF4G. (B) Radioactively labeled 71 nucleotide RNA at 1nM was incubated for 10 minutes with increasing amounts of eIF4G at 19°C and loaded onto a non-denaturing PAGE. (C) Quantification of gel from panel A. The data was fit to a hill equation (K_D = 123 ± 23nM, n = 2.0 ± 0.4)
Figure 3.6. RNA lengths determines affinity for eIF4G. (A) Binding competition scheme. A 2000 fold excess of RNAs ranging in size between 16-64nt (grey) were incubated with labeled 71nt RNA (black) and eIF4G. (i) If the competing RNA did not compete for eIF4G binding, then eIF4G bound the labeled 71nt RNA. (ii) When the competing RNA was long enough, no binding of the labeled RNA was observed. (B) 1nM of radioactively labeled 71 nucleotide RNA and unlabeled RNAs of various lengths were incubated at 19°C with 300nM eIF4G for 10 minutes, loaded onto a non-denaturing PAGE, and the bound fraction was quantified and plotted against the length of the competitor RNA. A trend line was added to fit the data.
dehydrogenase (LDH) and the substrate phosphoenolpyruvate (PEP) (106). The reduction of NADH absorbance is directly proportional to the amount of ATP hydrolyzed (106).

No significant change in NADH absorbance was observed for the Ded1p/eIF4G complex in the absence of RNA (Figure 3.7.A, filled circles). As expected, the absorbance decreased in the presence of Ded1p and RNA, indicating that Ded1p hydrolyzed ATP (Figure 3.7.A filled triangles). Addition of eIF4G to the RNA-stimulated Ded1p ATPase reaction did not change the amount of ATP hydrolysis (Figure 3.7.A, open triangles). No change in the ATP hydrolysis rate was detected even when eIF4G was in 4-fold excess over Ded1p (Figure 3.7.B). These data indicate that eIF4G does not enhance the RNA-stimulated ATPase activity of Ded1p.

3.2.6: eIF4G INHIBITS Ded1p HELICASE ACTIVITY IN VITRO.

Next, we tested whether eIF4G modulated Ded1p’s helicase activity. We added eIF4G to Ded1p helicase reactions and monitored changes in the rate constant for strand separation of a substrate containing a 13 bp duplex with a 3’ 25 nt ssRNA overhang (Figure 3.8.A). In the absence of eIF4G, Ded1p unwound the duplex to ~90% in 5 minutes (Figure 3.8.B, upper panel). When eIF4G was present, a noticeable decline in unwinding rate and amplitude was observed (Figure 3.8.B, lower panel). Ded1p alone readily unwound the RNA with an apparent first order rate constant of $k_{obs} = 1.15 \pm 0.06 \text{ min}^{-1}$ as expected (Figure 3.8.C, filled circles). The addition of eIF4G to the Ded1p unwinding reaction decreased the observed first order rate constant to $k_{obs} = 0.48 \pm 0.04 \text{ min}^{-1}$ (Figure 3.8.C,
Figure 3.7.

**Figure 3.7.** eIF4G does not stimulate Ded1p RNA-dependent ATPase activity. (A) The ATPase activity of 100nM Ded1p was monitored using a coupled ATP/NADH system in the absence of RNA (filled circle), in the presence of RNA (filled triangle), or in the presence of RNA and 400nM eIF4G (open triangle). (B) Observed ATPase rates of 100nM Ded1p plotted against eIF4G concentration.
Figure 3.8. eIF4G inhibits the helicase activity of Ded1p. (A) Schematic and sequence of RNA used in unwinding reaction. (B) 100nM Ded1p was incubated with 0.5nM of labeled RNA duplex in the presence or absence of 300nM eIF4G. Reactions were then started with 2mM (final) ATP/MgCl₂, and remodeling of the RNA was monitored by observing strand separation on a non-denaturing PAGE. (C) Quantification of upper panel (filled circle) and lower panel (open circle) from panel A. Data were fit to as described in (45) (k_{unw\text{-}eIF4G} = 0.97 ± 0.05 \text{ min}^{-1}, k_{ann\text{-}eIF4G} = 0.52 ± 0.07 \text{ nM}^{-1}\text{min}^{-1}, k_{unw\text{+eIF4G}} = 0.17 ± 0.01 \text{ min}^{-1}, k_{ann\text{+eIF4G}} = 0.90 ± 0.07 \text{ nM}^{-1}\text{min}^{-1})
open circles). In addition, the presence of eIF4G reduced the amplitude of the unwinding reaction by ~2.5 fold (Figure 3.8.C).

Ded1p is known to catalyze both duplex unwinding and strand annealing, and the steady state between those two opposing activities determines the reaction amplitude (64). Thus, if the ratio between unwinding and annealing rates changes, the reaction amplitude changes as well. To examine whether a new steady state between unwinding and annealing was established in the presence of eIF4G, we compared the reaction amplitudes for both unwinding and annealing reactions with Ded1p and eIF4G. Annealing reactions were measured under conditions identical to those in the strand separation reaction, except that reactions were started with single stranded RNAs. When we tested the annealing with Ded1p and eIF4G, identical reaction amplitudes were observed for both unwinding and annealing reactions (Figure 3.9). These findings indicated that a steady state was reached in the presence of eIF4G. The reduction in amplitude suggested that increased annealing compared to the unwinding reaction, caused the decrease in the reaction amplitude. When eIF4G was incubated with heat denatured duplex, eIF4G accelerated the spontaneous rate constant for annealing and completely annealed the RNA in 30 min. (Figure 3.10), indicating that eIF4G possesses considerable strand annealing activity.

We next examined the cause for the eIF4G-dependent decrease in the observed rate constant for the unwinding reaction. To this end we performed unwinding reactions with increasing concentrations of eIF4G and monitored the kinetics of strand separation. The
Figure 3.9. The decrease in amplitude is due to strand annealing catalyzed by eIF4G. Representative time-course of 100nM Ded1p and 300nM eIF4G catalyzed annealing (filled circles) and unwinding (open circles). Data from the reaction starting from denatured RNA strands was fit as described (64) ($k_{\text{unw}} = 0.12 \pm 0.01 \text{ min}^{-1}$, $k_{\text{ann}} = 0.33 \pm 0.04 \text{ nM}^{-1}\text{min}^{-1}$). Kinetic data for reaction starting from duplex RNA is reported in Figure 3.8.
Figure 3.10. eIF4G possess strand annealing activity. Representative time-course for strand annealing without (filled circles) or with 300nM eIF4G (open circles). Annealing data was fit to the integrated rate law for the bimolecular annealing reaction, considering that both strands were present at equal concentrations. $k_{\text{ann}^-\text{eIF4G}} = (3.9 \pm 0.3) \times 10^{-2} \text{nM}^{-1}\text{min}^{-1}$ and $k_{\text{ann}^+\text{eIF4G}} = (7.3 \pm 1.4) \times 10^{-1} \text{nM}^{-1}\text{min}^{-1}$. 
observed rate constant ($k_{\text{obs}}$) for the unwinding reactions were mathematically separated into apparent first order unwinding rate constants ($k_{\text{unw}}$) and apparent second order annealing rate constants ($k_{\text{ann}}$) (64). The unwinding rate constants decreased in an eIF4G concentration dependent manner and then plateaued at ~0.18 min$^{-1}$ (Figure 3.11.A). As expected, the annealing rate constants increased with increasing eIF4G concentrations until leveling at ~1.0 nM$^{-1}$min$^{-1}$ (Figure 3.11.B). Interestingly, the ~1.7 fold increase in the annealing rate constant does not match the ~5.6 fold decrease in unwinding rate constant, suggesting that Ded1p unwinding inhibition is not solely due to the increase in eIF4G annealing.

Addition of eIF4G to unwinding reactions with the DEAD-box protein Mss116p did not cause any inhibition (Figure 3.12). These data suggest that the eIF4G-dependent inhibition seen with Ded1p is not due to competition for the RNA. This notion is further supported by the plateau seen in the inhibition of the Ded1p unwinding reactions. The unwinding rate constants would not level if eIF4G were inhibiting Ded1p unwinding through simple binding competition for the RNA. Instead, the unwinding rate constants would decrease to zero with increasing eIF4G concentrations.

To further verify that the eIF4G dependent inhibition of Ded1p was not due to competition for ssRNA binding, we monitored the kinetics of unwinding of a 13 bp blunt ended duplex. If eIF4G was sequestering the ssRNA region and prevented loading of Ded1p (18), then Ded1p would have to initiate unwinding directly from the duplex. Consequently, the observed unwinding rate constants of the blunt duplex and the duplex
Figure 3.11. eIF4G inhibits Ded1p strand separation. (A) Observed $k_{\text{unw}}$ rate constants at 100nM Ded1p measured at various concentrations of eIF4G. (B) Observed $k_{\text{ann}}$ rate constants at 100nM Ded1p measured at various concentrations of eIF4G.
**Figure 3.12.** eIF4G does not inhibit Mss116p strand separation. $k_{unw}$ rate constants at 5nM Mss116p measured at various concentrations of eIF4G.
with a ssRNA region would be similar if eIF4G was present. However, no significant
differences were observed between Ded1p unwinding of the 13 bp blunt duplex in the
presence and absence of eIF4G, which is expected since eIF4G can not bind RNAs
smaller than 21 nt (Figure 3.13). With eIF4G, Ded1p unwound the blunt 13 bp duplex
with a first order rate constant of \( k_{\text{obs}} = (2.4 \pm 0.3) \times 10^{-3} \text{ min}^{-1} \). The blunt duplex
unwinding rate constant was 2 orders of magnitude smaller than the unwinding rate
constant for the duplex with a ssRNA overhang (Figure 3.13). Even when the
concentration of Ded1p was increased 3-fold, the blunt duplex unwinding rate constant
was still lower than the rate constant seen for the duplex with the ssRNA region (Figure
3.13). These data indicate that eIF4G did not sequester the entire ssRNA region. It is,
however, possible that the presence of eIF4G decreases the number of ssRNA binding
sites available for Ded1p to bind. As a result, eIF4G could interfere with the single
strand mediated loading of Ded1p onto the duplex, which could slow the rate of
unwinding.

3.2.7: Ded1p AND eIF4G SYNERGISTICALLY STABILIZE RNA BINDING

The observations made while examining Ded1p strand separation in the presence of
eIF4G suggested that eIF4G affected the interaction of Ded1p with the RNA. To further
investigate effects of eIF4G on the interaction of Ded1p with RNA, we first monitored
whether eIF4G altered the binding of Ded1p to a 35 nt RNA. Both Ded1p and eIF4G
alone did not bind the RNA to a significant degree at sub-saturating concentrations
(Figure 3.14.A, lanes 2 and 3). However, combining eIF4G and Ded1p increased the
amount of ssRNA bound. Furthermore, we observed a band migrating more slowly than
Figure 3.13. eIF4G inhibition of Ded1p is not due to complete binding competition. Observed unwinding rate constants at either 100nM (+) or 300nM (+++) Ded1p in the absence (filled circles) or presence (open circles) of 300nM eIF4G for duplexes with and without single stranded regions.
the eIF4G/RNA band, suggesting that both eIF4G and Ded1p were bound to the same RNA (Figure 3.14.A, lane 4). When the RNA binding was performed in the presence of ATP, no change in the fraction of RNA bound was observed (data not shown). Thus, Ded1p did not displace eIF4G from the RNA in the presence of ATP, consistent with the notion that Ded1p cannot remove proteins with a large binding site size from RNA (98). Most importantly, the observations suggested that the Ded1p/eIF4G interaction stabilizes both proteins on RNA.

To analyze the synergistic RNA binding effect observed between Ded1p and eIF4G, we measured to what degree Ded1p increased the affinity of eIF4G for RNA. We incubated increasing amounts of eIF4G with the 35 nt ssRNA in the absence or presence of Ded1p (Figure 3.14.B). High concentrations of eIF4G (>100nM) were required to see appreciable binding of the RNA (Figure 3.14.B, upper panel). In the presence of 100nM Ded1p, however, RNA binding was observed at lower concentrations of eIF4G (Figure 3.14.B, lower panel). When directly comparing RNA binding in the presence and absence of Ded1p, a 23-fold increase in binding was detected at an eIF4G concentration of 7.5nM. While eIF4G bound to the RNA with an equilibrium dissociation constant of $K_D = 77.0 \pm 7.5 \text{ nM}$, the presence of Ded1p decreased it to $K_D = 25.4 \pm 2.6 \text{ nM}$ (Figure 3.14.C). In fact, increasing Ded1p concentrations in the RNA binding reactions lowered the equilibrium dissociation constant for the Ded1p/eIF4G complex for RNA (Figure 3.14.D). The data thus clearly show that, Ded1p increases eIF4G’s affinity for RNA.
Figure 3.14. Ded1p increases eIF4G’s affinity for RNA. (A) An EMSA was performed with 1nM of a 35 nucleotide long RNA incubated with 100nM Ded1p, 50nM eIF4G, or 100nM Ded1p and 50nM eIF4G. (B) The RNA from panel A was incubated for 10 minutes with increasing amounts of eIF4G in the presence or absence of 100nM Ded1p at 19°C and loaded onto a non-denaturing PAGE. (C) Representative binding curves in the presence (open circlces) or absence (filled circles) of 100nM Ded1p. Data was fit with the hill equation ($K_D^{-eIF4G} = 73 \pm 7$ nM, $K_D^{+eIF4G} = 25 \pm 3$ nM). (D) Observed RNA binding $K_D$ for eIF4G/Ded1p in the presence of various concentrations of Ded1p. A trend line was added to fit the data.
3.2.8: Ded1p GREATLY FACILITATES eIF4G BINDING TO SHORT RNAs.

We next tested whether Ded1p could also stabilize eIF4G on short RNAs that eIF4G by itself was unable to bind to an appreciable degree. We measured eIF4G binding to a 16nt ssRNA. Without eIF4G, Ded1p bound to RNA visible as one band in a non-denaturing PAGE (Figure 3.15.A, lane 2). To obtain these Ded1p/RNA complexes, binding was performed in the presence of ADPNP. As expected without Ded1p, no binding of eIF4G to this RNA was seen (Figure 3.15.A, lane 3). In the presence of both Ded1p and eIF4G, two bands were visible, one migrated with the Ded1p-bound RNA species, and the other band migrated more slowly, consistent with a complex formed by Ded1p and eIF4G on the RNA (Figure 3.15.A, lane 4). Notably, both RNA species were observed despite the presence of a large excess of 73 nt scavenger RNA. Thus, the putative Ded1p/eIF4G-RNA complex appeared to be highly stable.

To determine which of the shifted bands in the EMSA experiments contained Ded1p, eIF4G, or both, we utilized antibodies that targeted Ded1p and eIF4G (Figure 3.15.A, lane 4). As a control, we incubated Ded1p bound to the 16 nt long RNA with an antibody against Ded1p (pAb α-Ded1p, Figure 3.15.B, left panel). The Ded1p/RNA complex was super-shifted to the well, indicating that the antibody bound Ded1p associated to RNA. When α-Ded1p was added to a binding reaction containing Ded1p and eIF4G, both bands were shifted to the well, implying that Ded1p bound to both complexes (Figure 3.15.B, right panel). When a pAb against the C-terminus of eIF4G (α-eIF4G) was added, the slower migrating band disappeared, while the signal of the remaining band intensified. The absence of the slower species suggested that α-eIF4G
prevented eIF4G association to the Ded1p/RNA complex (Figure 3.15.B, right panel). Furthermore, addition of α-S Tag against the N-terminus of eIF4G redistributed a portion of the eIF4G bound species to the well, too (Figure 3.15.B, right panel). An antibody that is not specific for Ded1p or eIF4G did not shift either species (Figure 3.15.B, right panel). These data suggested that the slower migrating band in the Ded1p/eIF4G/RNA binding reactions represented a complex of Ded1p/eIF4G on the RNA. This observation implies that Ded1p greatly enhances binding of eIF4G to RNAs that it otherwise cannot bind to any appreciable degree.

Interestingly, the antibody against the C-terminus but not the N-terminus of eIF4G inhibited formation of the Ded1p/eIF4G/RNA complex. These data raised the possibility that α-eIF4G competes with Ded1p for binding to eIF4G’s C-terminus. Alternatively, α-eIF4G may produce a conformational change in eIF4G structure preventing Ded1p association with eIF4G.

To verify the identity of the Ded1p/eIF4G/RNA complex, we incubated increasing amounts of eIF4G with preformed Ded1p/RNA complex and determined the fraction of RNA bound by non-denaturing PAGE. The appearance of the slower migrating band was observed in an eIF4G concentration dependent manner (Figure 3.15.C), confirming that this species was a result of eIF4G association.

To determine the rate of dissociation of eIF4G from the complex, we monitored by non-denaturing PAGE the dissociation of eIF4G from the preformed
Figure 3.15. Ded1p greatly increases eIF4G’s affinity for 16 nt RNA. (A) 1nM of a 16 nucleotide long RNA and 2mM ADPNP was incubated at 19°C for 1.5 hours in the presence or absence of 100nM Ded1p. Reactions were then incubated for 10 minutes with or without 300nM eIF4G, challenged with 1µM of competitor RNA for 1 minute, and loaded onto a non-denaturing PAGE. (B) Binding reactions as performed in panel A with the addition of antibodies. (C) Binding reactions as performed in panel A with various concentrations of eIF4G. Data was fit with the hill equation ($K_D = 98 \pm 4$ nM, $n = 1.6 \pm 0.1$).
eIF4G/Ded1p/ADPNP/RNA complex after challenge with a high concentration of unlabeled competitor (Figure 3.16.A). eIF4G dissociated from the complex with an apparent first order rate constant of $k_{\text{dissoc.}} = (7.2 \pm 0.1) \times 10^{-4}$ min$^{-1}$ (Figure 3.16.B). These data showed that eIF4G bound tightly and dissociated slowly from the RNA-bound Ded1p.

3.2.9: eIF4E DOES NOT BIND TO Ded1p

As mentioned before, the eIF4G was purified as a complex with the mRNA cap binding protein eIF4E. Although genetic data suggest that Ded1p and eIF4E do not interact (104) and no other evidence of such an interaction exists, it was important to probe specifically whether or not the interaction between eIF4G and Ded1p was due to binding between eIF4E and Ded1p. To determine if eIF4E bound Ded1p, we first purified His-tagged eIF4E from *E. coli*. We next examined if the purified eIF4E retained its physiological function by measuring eIF4E binding to the mRNA cap analog ARCA (Anti-Reverse Cap Analog). ARCA binding was monitored through changes in tryptophan fluorescence of eIF4E (107). As increasing concentrations of ARCA cap were added, the tryptophan fluorescence of eIF4E decreased, as expected for functional eIF4E (Figure 3.17). We therefore concluded that the purified eIF4E was folded correctly and functional.

We then probed binding of eIF4E to Ded1p through a functional competition assay. If eIF4E was capable of binding Ded1p, then purified eIF4E should compete with eIF4G/4E for binding to the Ded1p/RNA complex. For this competition experiment, we formed the
Figure 3.16. eIF4G tightly associates with Ded1p/ADPNP/RNA complex. (A) A labeled 16nt RNA was incubated at 19°C for 1.5 hours with 100nM Ded1p and 2mM ADPNP. The mixture was then incubated for 10 minutes with eIF4G. The preformed complex was challenged with unlabeled Ded1p/ADPNP/RNA complex. Aliquots were removed over time and loaded onto a non-denaturing PAGE. (B) Data from A normalized to the initial amount of free RNA was fit to first order kinetics \[ k_{\text{off}}^{\text{eIF4G}} = (7.2 \pm 0.1) \times 10^{-4} \text{ min}^{-1} \].
Figure 3.17. Purified eIF4E binds the mRNA cap analog ARCA. Purified eIF4E (500nM) was incubated with various concentrations of ARCA; 0μM (solid), 100μM (dashed), and 400μM (dotted). The tryptophan fluorescence of the eIF4E mixtures excited at 295nm was then measured between 315-400nm. Background fluorescence from the buffer and ARCA cap were subtracted out.
eIF4G/Ded1p/RNA complex in the presence of eIF4E and monitored changes in the fraction of eIF4G bound RNA (Figure 3.18.A). No change in eIF4G association with the Ded1p/RNA complex was observed over a range of eIF4E concentrations (Figure 3.18.B). Additionally, in the absence of eIF4G, eIF4E did not retard any Ded1p/RNA complexes at any concentration used (data not shown). These data indicated that eIF4E does not bind to Ded1p, and that the shifted species observed in the presence of eIF4G/4E was solely due to eIF4G.

3.3: DISCUSSION

Here, we have identified a protein-protein interaction between eIF4G and the DEAD-box protein Ded1p. Although the exact role of Ded1p during translation still remains unclear, Ded1p’s interaction with eIF4G positions Ded1p early in the translation initiation scheme. This notion is consistent with previous studies which had indirectly linked Ded1p with steps in translation initiation that require eIF4G (108). Berthelot et al suggested that Ded1p was required to melt mRNA secondary structure during 43S ribosomal subunit scanning (108). Scanning is the process during translation initiation where the 43S ribosomal subunit migrates along mRNA until it reaches the initiating AUG start codon (46). Although we cannot confirm Ded1p’s involvement in scanning, Ded1p could indirectly associate with the 43S ribosomal subunit through Ded1p’s interaction with eIF4G. It is noteworthy, however, that we did not identify any ribosomal proteins from our immunoprecipitations of Ded1p with the mAb α-HA (Figure 3.2), suggesting that Ded1p does not tightly associate with the ribosome.
Figure 3.18. eIF4E does not compete for binding of Ded1p. (A) A 16nt RNA and ADPNP was incubated at 19°C for 1.5 hours in the presence or absence of 100nM Ded1p. The mixtures were then incubated for 10 minutes with 300nM eIF4G in the presence or absence of 400nM eIF4E, challenged with a competitor RNA for 1 minute, and loaded onto a non-denaturing PAGE. (B) Competition experiments as in A performed under various concentrations of eIF4E.
Our work focused on the mechanistic implications of the Ded1p/eIF4G interaction. Despite the clear binding of eIF4G to Ded1p, eIF4G did not stimulate Ded1p’s ATPase or helicase activities \textit{in vitro}. We observed an eIF4G-dependent inhibition of Ded1p’s helicase activity. However, it remains unclear whether the decrease in unwinding was a result of the interaction between Ded1p and eIF4G or partial RNA binding competition between the proteins for the single stranded region of the RNA. Although ATPase and helicase activities of several DEAD-box proteins are stimulated by other proteins (5, 27, 50, 51, 58), there is also precedent for interactions between DEAD-box proteins without effects on ATPase or helicase activities (59). The interaction between UAP56 and U2AF\textsuperscript{65} has no effects on UAP56’s ATPase or helicase activities (59). However, genetic evidence indicates that UAP56 is recruited by U2AF\textsuperscript{65} so that UAP56 can remove proteins from the branch point site during splicing (29, 98).

The lack of eIF4G-dependent stimulation of Ded1p helicase activity suggests that the interaction between Ded1p and eIF4G does not serve to facilitate unwinding of heavily structured RNAs during translation. However, it does not rule out that Ded1p by itself may unwind mRNA structure. It is possible that Ded1p recruits eIF4G to mRNA during translation initiation. Alternatively, Ded1p could be bound to mRNAs and recruit eIF4G during translation initiation. This notion is consistent with our data showing that Ded1p stabilizes eIF4G binding to RNA, even if the RNA is too small for eIF4G to bind alone.

Our results suggest a basic mechanism by which Ded1p influences eIF4G RNA binding (Figure 3.19). If a stretch of RNA 35 nt or longer is available, eIF4G can bind the RNA
by itself. Nonetheless, subsequent binding by Ded1p stabilizes eIF4G on the RNA (Figure 3.19.A, pathway 1 & 2), although the stabilization appears moderate in energetic terms. Alternatively, Ded1p could bind the RNA first, followed by eIF4G binding to the RNA resulting in the more stable Ded1/eIF4G/RNA complex (Figure 3.19.A, pathway 3 & 4). However, if only a few nucleotides (≤21 nt) of RNA are available for eIF4G association, eIF4G can not bind by itself. Recruitment of eIF4G to the RNA can then occur through binding of Ded1p to the RNA, followed by eIF4G association to Ded1p (Figure 3.19.B, pathway 3 &4).

The ability of Ded1p to facilitate eIF4G RNA binding is another example of a DEAD-box protein functioning to load/recruit ancillary RNA binding proteins onto RNA. UAP56 facilitates in the loading of the splicing factor Aly/Ref onto intronless RNAs (60). Aly/Ref helps recruit mRNA export factors assisting the export of RNA out of the nucleus (109). Ded1p however, did not load eIF4G in an ATP-dependent fashion on RNAs longer than 35 nt, indicating that Ded1p does not function exactly like UAP56. It is possible that Ded1p functions more like eIF4AIII, which recruits proteins to RNA through protein-protein interactions while remaining bound to RNA (26, 28). eIF4AIII stabilizes the exon junction complex (EJC) on mRNA (5, 28). Through inhibition of eIF4AIII’s ATP hydrolysis, eIF4AIII binds and does not release mRNA until hydrolysis of ATP occurs (5, 28). eIF4AIII associates with a number of proteins in the EJC (26, 28) which confines the EJC to the RNA in an ATP-dependent fashion. In a similar manner, Ded1p locked onto a 16 nt RNA by the non-hydrolysable ATP analog ADPNP was capable of binding eIF4G. When ADPNP was not present, eIF4G was not loaded onto
Figure 3.19. Model for Ded1p-mediated enhancement of eIF4G RNA binding. (A) Ded1p and eIF4G binding of a long stretch of RNA. eIF4G may bind first (i) followed by Ded1p (ii), or Ded1p may go first (iii) followed by eIF4G (iv). (B) Ded1p and eIF4G binding of a short stretch of RNA. eIF4G may only bind if Ded1p binds first (iii, iv).
the 16nt RNA (data not shown). The nucleotide dependence and requirement of a RNA bound Ded1p for eIF4G recruitment thus appears to mirror more closely the eIF4AIII-like RNA clamp model than the UAP56-like loader model. It is interesting to speculate that Ded1p functions as an ATP dependent RNA clamp during translation initiation, although other factors would be required to inhibit the ATP hydrolysis reaction by Ded1p. Notwithstanding, our observations do not unequivocally discern the role of Ded1p during translation initiation. It also is possible that a complex containing more than just eIF4G and Ded1p may have different effects on Ded1p’s ATPase and helicase activities from those seen with eIF4G. However, the absence of eIF4G-dependent stimulation of the ATPase and helicase activities of Ded1p was unexpected, especially since eIF4G has been shown to stimulate another DEAD-box protein, eIF4AI (44, 55). We also demonstrated that DEAD-box proteins can use their RNA and ATP binding activities to modulate the RNA binding of other proteins thereby becoming cofactors themselves. As more studies are conducted, it will be interesting to see if this is a common theme for all or just a portion of DEAD-box proteins.
CHAPTER 4: FUTURE DIRECTIONS

Here we identified several potential Ded1p cofactors, including eIF4G. We examined eIF4G as a potential Ded1p cofactor because an eIF4G/Ded1p interaction had already been established (103) and eIF4G was genetically linked to Ded1p (104) placing a physiological emphasis on this interaction. We found that eIF4G inhibits Ded1p helicase activity most likely by affecting the loading of Ded1p onto RNA. Interestingly, Ded1p greatly increases eIF4G’s affinity for RNA even to RNAs too small for eIF4G to bind by itself. These observations helped us develop a basic model for Ded1p enhancement of eIF4G RNA binding \textit{in vitro}. However, determining if the physiological importance of the Ded1p/eIF4G interaction is related to RNA binding and characterizing further interactions we identified would potentially provide more insight into Ded1p’s role in translation.

4.1: DETERMINING THE PHYSIOLOGICAL IMPORTANCE OF THE eIF4G/Ded1p INTERACTION

To determine the role of the eIF4G/Ded1p interaction during translation, we propose to monitor the phenotype caused by disrupting the eIF4G/Ded1p interaction through site-directed mutagenesis. We can then rescue the phenotype by restoring the interaction by exogenously adding wildtype Ded1p or eIF4G. Additionally, we can add mutant Ded1p deficient in helicase, ATPase, or RNA-binding and determine which activities are required for translation.
To disrupt the interaction between eIF4G and Ded1p, we must first determine where Ded1p binds eIF4G and *vice versa*. Preliminary data suggests Ded1p binds within the first 164 amino acids of eIF4G (Figure 4.1). These data were obtained with GST or GST-eIF4G constructs (Figure 4.1.A) from *E. coli* lysates. The constructs were immobilized on glutathione-sepharose resin, washed, and incubated with purified Ded1p. BSA was also added to prevent non-specific binding. After several washes, proteins were resolved by SDS-PAGE and western blot analysis was conducted using an antibody specific for Ded1p (pAb α-Ded1p). GST did not pulldown Ded1p, consistent with previous results (Figure 4.1.B lane 1, cf Figure 3.2.B). In contrast, Ded1p was present in every lane where a lysate containing a GST-eIF4G construct was used, including the lysate containing GST-eIF4G 1-164 (Figure 4.1.B, lanes 2-5). These data further support the notion that Ded1p does not bind eIF4E and rules out that Ded1p competes with eIF4A for binding to eIF4G since the first 164 amino acids of eIF4G does not contain the eIF4E or eIF4A binding sites. To confirm these results, we propose performing a GST-pulldown with an eIF4G construct containing amino acids 165-952. The absence of Ded1p binding to the eIF4G165-952 construct would confirm that Ded1p binds to the first 164 amino acids of eIF4G. However, if Ded1p does associate with the eIF4G165-952 construct, then the results would suggest that Ded1p binds eIF4G at two sites.

Once the binding sites for Ded1p and eIF4G have been determined on their interacting partner, mutations can be designed to disrupt the interaction. For example, a deletion of the amino acids in Ded1p responsible for binding eIF4G should prevent eIF4G binding to Ded1p. GST-pulldowns can confirm the expected result. This mutation can
Figure 4.1

(A) Schematic representation of full length (FL) and fragments of GST-eIF4G used for GST-pulldowns. RBD, PABP, 4E, and 4A signify RNA-binding domain, the binding site of poly-A binding protein, eIF4E, and eIF4A on eIF4G respectively. (B) eIF4G constructs from bacterial lysates were immobilized on glutathione-sepharose resin and washed. Purified Ded1p (80nM) and BSA (0.5% w/v) were incubated with the resin and washed. Samples were loaded onto a 8-15% SDS-PAGE, probed with α-Ded1p, and then coomassie stained. * represents the band that corresponds to the estimated size of the GST constructs.

Figure 4.1. Ded1p binds to the first 164 amino acids of eIF4G. (A) schematic representation of full length (FL) and fragments of GST-eIF4G used for GST-pulldowns. RBD, PABP, 4E, and 4A signify RNA-binding domain, the binding site of poly-A binding protein, eIF4E, and eIF4A on eIF4G respectively. (B) eIF4G constructs from bacterial lysates were immobilized on glutathione-sepharose resin and washed. Purified Ded1p (80nM) and BSA (0.5% w/v) were incubated with the resin and washed. Samples were loaded onto a 8-15% SDS-PAGE, probed with α-Ded1p, and then coomassie stained. * represents the band that corresponds to the estimated size of the GST constructs.
be introduced into a ded1 null yeast strain to obtain a phenotype. The phenotype may manifest itself as a growth defect, or as a deficiency in ribosome assembly which can be determined by ribosome profiles (23).

If deletion of the eIF4G binding site on Ded1p results in a non-viable phenotype, two copies of Ded1p should be introduced; a Ded1p copy containing the eIF4G site and one without it. With this approach, we can determine which biochemical activities of Ded1p (i.e. RNA-binding, ATPase, and helicase) are required for efficient translation. One plasmid will contain a functional copy of Ded1p lacking the eIF4G binding site to perform all of Ded1p’s functions that do not involve eIF4G. Additionally, we can transform a RNA binding-deficient copy of Ded1p containing the eIF4G binding site to determine if RNA binding is required for translation. The same procedure can be used to determine if the ATPase and helicase activities of Ded1p are required for translation. The information obtained from these experiments will not only provide the physiological importance for the eIF4G/Ded1p interaction, but will also offer details about the activities required of Ded1p during translation.

4.2: Ded1p AND NUCLEAR EXPORT OF mRNA

Ded1p and its orthologs have been implicated in nuclear export of mRNA (68, 84, 86). Ded1p interacts with Crm1p and Npl3p, two proteins involved in independent mRNA export pathways (110). Crm1p, chromosome region maintenance 1, is an importinβ-like transport factor that interacts with the NES motif and Ran GTPase (85). Npl3p, nuclear
protein localization, contains 2 RNA recognition motifs (RRM) and a Gly-Arg repeat domain that binds mRNA for shuttling from the nucleus to the cytoplasm (111, 112).

4.2.1: Ded1p AND THE EXPORT FACTOR CRM1p

The Ded1p orthologs An3 and DDX3 from *X. laevis* and *Homo sapien* both utilize the Crm1p pathway to shuttle from the nucleus to the cytoplasm (68, 84). Crm1p binds the conserved NES (Figure 4.2.A) and exports the Ded1p orthologs (68). An3 helicase activity is required for export from the nucleus consistent with a scenario where An3 functions to unwind RNA or remodel an RNP during export (87). Despite these results, export of Ded1p via the Crm1p pathway has never been observed. Furthermore, immunofluorescence and GFP imaging of Ded1p has suggested that Ded1p localizes to the cytoplasm (23, 88).

To evaluate the function of the NES in Ded1p, we transformed a copy of Ded1p containing two point mutations in the NES motif (L11A/I13A) and a C-terminal HA-tag into yeast. Overexpression of wildtype Ded1p with the HA-tag showed a decrease in growth when compared to the LacZ control as expected (88). However, the Ded1p NES mutant had a more pronounced growth defect than the wildtype (Figure 4.2.B) and the doubling time of the Ded1p NES mutant increased (data not shown). Furthermore, a haploid yeast strain harboring only one copy of Ded1p with a mutated NES signal was not viable indicating that a functioning Ded1p NES is important for growth (data not shown).
Figure 4.2

(A) Conserved N-terminal NES sequence in the Ded1p family of RNA helicases is revealed by alignment of the amino termini of the Ded1p’s orthologs. The proposed leucine-rich NES consensus sequence is shown at the bottom. The Φ sign denotes amino acids M, V, I, L, F, or W. Yellow bars represent the amino acids mutated to alanine. Figure was adapted from reference (68).

(B) Constructs driven by a galactose promoter containing either HA tagged LacZ, wildtype Ded1p, or L11A/I13A Ded1p were transformed into the yeast strain BY4741. The yeast were grown to an OD$_{600}$ of ~1, serial diluted, and 10µl of each dilution was placed on a YPG plate. The yeast were grown at 30°C for 2 days.

Figure 4.2. Disruption of the conserved NES in Ded1p slows growth. (A) Conserved N-terminal NES sequence in the Ded1p family of RNA helicases is revealed by alignment of the amino termini of the Ded1p’s orthologs. The proposed leucine-rich NES consensus sequence is shown at the bottom. The Φ sign denotes amino acids M, V, I, L, F, or W. Yellow bars represent the amino acids mutated to alanine. Figure was adapted from reference (68). (B) Constructs driven by a galactose promoter containing either HA tagged LacZ, wildtype Ded1p, or L11A/I13A Ded1p were transformed into the yeast strain BY4741. The yeast were grown to an OD$_{600}$ of ~1, serial diluted, and 10µl of each dilution was placed on a YPG plate. The yeast were grown at 30°C for 2 days.
To confirm that the mutated NES causes nuclear localization of Ded1p, we can monitor the immunofluorescence of Ded1p utilizing antibodies against the C-terminal HA tag. In addition, the yeast can be divided into nuclear and cytoplasmic factions to determine the localization of Ded1p with a mutated NES by western blot analysis (68). These results will probe whether Ded1p functions in the nucleus as suggested by its orthologs.

**4.2.2: Ded1p AND THE EXPORT FACTOR NPL3p**

Two previous studies and our work have identified Npl3p as an interacting partner of Ded1p (86, 113). Npl3p is recruited to nascent mRNAs via interactions with RNA polymerase II (113) to protect the mRNA from premature transcription termination, and also to package the fully processed transcript for export (112). Ded1p co-immunoprecipitated with RNA polymerase II presumably through Npl3p (113). Yeast two hybrid experiments have also confirmed that Ded1p and Npl3p interact, but only when the NES motif in Ded1p has been mutated indicating that increased nuclear concentrations of Ded1p are required for Yeast 2 hybrid analysis (Gao, Z. unpubl.). We propose examining the effects of the interaction on Ded1p’s biochemical activities and nuclear function(s).

We intend to map the interactions sites between each protein. If the interaction serves to inhibit or stimulate Ded1p’s biochemical processes, Npl3p should bind somewhere within the core motor domain of Ded1p as Magoh/Y14 does with eIF4AIll (28). If the interaction serves to recruit Ded1p to nascent mRNAs, Npl3p should bind either on the
unstructured N or C–terminal ends of Ded1p. It is also possible that binding of Npl3p to the termini of Ded1p causes conformational changes within Ded1p to facilitate RNA binding. However, eIF4G increased the stabilization of Ded1p on RNA and no stimulation of ATPase or helicase activity was observed (Figure 3.7 and 3.8). Npl3p also contains unstructured N and C termini, while the core of the protein consists of two RNA recognition motifs (RRMs) (112). It is unclear whether Ded1p contacts the RRM5s or the unstructured termini. After determining the interaction sites, we plan to examine the biochemical and physiological effects of the interaction.

To determine if Npl3p affects Ded1p’s biochemical activities, full length and a fragment of Npl3p that interacts with Ded1p will be purified from E. coli. The RNA binding, ATPase, and helicase activities of Ded1p will be tested with and without Npl3 (full length and fragment). To acquire physiological information about the interaction, we will disrupt the interaction between Npl3p and Ded1p. By overexpressing the Npl3p fragment that binds to Ded1p in yeast, we can prevent Ded1p binding to Npl3p. The result will most likely resemble that of a dominant negative phenotype. The fragment may not contain a NES, in which case we will have to engineer one on the construct. These results will help define detailed mechanistic parameters for the interaction between Ded1p and Npl3p.

Interestingly, it has been independently demonstrated that Kem1p/Xrn1p also binds to Ded1p and Npl3p (86, 113). Kem1p/Xrn1p is a 5’→3’ exonuclease implicated in rRNA biogenesis and the mRNA decay found in P-bodies (114, 115). It is intriguing to
speculate that Ded1p, Npl3p, and Kem1p form an EJC-like complex that associates with RNA in the nucleus, is exported, and then performs additional functions in the cytoplasm. To investigate this possibility, we propose co-immunoprecipitating Ded1p from nuclear and cytoplasmic extracts followed by Npl3p co-immunoprecipitations. Using nuclear and cytoplasmic extracts allows for identification of Kem1p association with Npl3/Ded1p complexes at different stages of mRNA processing. Differences between compositions of the complexes will serve to influence our next course of action.

4.3: INTERACTIONS BETWEEN GLYCOLYTIC PROTEINS AND Ded1p

The majority of the metabolism proteins identified in our Ded1p co-immunoprecipitation experiment (Figure 3.2) belonged to two of the most well understood metabolic pathways, glycolysis and the TCA cycle. In combination, glycolysis and the TCA cycle utilize the processing of one glucose molecule to add phosphates onto several molecules of ADP to produce many molecules of ATP. The connection between Ded1p interacting with proteins that synthesis ATP and Ded1p’s ability to hydrolyze ATP suggest that Ded1p may regulate the glycolysis and TCA cycles. Alternatively, these proteins could control their own protein levels through regulation of Ded1p and translation. Interestingly, two of the proteins Ded1p associates with, phosphofructokinase 2 (Pfk2p) and Pyruvate Kinase (Pyk1p), control rate-limiting steps in glycolysis.

To confirm the interaction between Pfk2p and Ded1p, we performed western blot analysis on immunoprecipitates of Ded1p. To elucidate whether Pfk2p and Ded1p interacted through RNA, we conducted immunoprecipitations in the presence and
Figure 4.3. Pfk2p co-immunoprecipitates with Ded1p in an RNA independent manner. Yeast lysates were incubated with α-HA beads to isolate HA tagged Ded1p. After washing, the beads were heat denatured along with the inputs and loaded onto 8% SDS page gels and probed with pAb Pfkp or pAb Ded1p.
absence of RNAse A (Figure 4.3). Pfk2p was only detected in the immunoprecipitations containing a HA tagged Ded1p, and RNAse A did not significantly affect the presence of Pfk2p or Ded1p. These data indicated that Ded1p and Pfk2p interact in a RNA independent manner either through other proteins or direct protein-protein contacts.

Having demonstrated an interaction between Pfk2p and Ded1p, we need to determine if there is a genetic link between Ded1p and Pfk2p and thus a physiological aspect for the interaction. By supplying an isogenic copy of the temperature sensitive Ded1p mutant (23) in a null ded1 and pfk2 background, we can determine if the double mutation results in a genetic enhancement (i.e. no growth) once the yeast are shifted to a non-permissive temperature. If no genetic interaction is observed, it would indicate that the interaction is not relevant. If the double mutation is lethal once shifted to a non-permissive temperature, it will suggest that the interaction between Ded1p and Pfk2p is physiologically important. Despite determining the relevance of the interaction between Ded1p and Pfk2p, it will be difficult to determine if the interaction is required for translation or sugar metabolism. Because Ded1p is required for translation initiation (23), any changes monitored in sugar metabolism or CO₂ release may be an indirect result of decreased protein production. Monitoring ribosome profiles and protein levels may determine if the observed data is a result of decreased translation.
MATERIALS AND METHODS

DNA CONSTRUCTS AND PLASMIDS

The pET22b(+) plasmid containing a N-terminally Histidine tagged Ded1p was a gift from Patrick Linder (Geneeeva, Switzerland) (65). This plasmid was referred to as pET22b+-Ded1.

The DED1 DNA construct used for homologous recombination was created through PCR amplification of yeast genomic DNA utilizing the primers, 5'-CCGTAGTGAGAGTGCGTTCAA and 5'-CACATATTGCTAGGCAACCC. The PCR product was gel purified, digested with Hind III and Pst I, and ligated into the plasmid pBlueScript II KS(+). A 3x HA tag was inserted with PCR amplification of the new plasmid with the primers 5'PO4-GCCCGCATAGTCAGGAACATCGTATGGGTTAGCCCGCATAGTCAGGAACATCGTATGGGTACCACCAAGAAGTTGTTTG and 5'PO4-TGATTTCAGACAAACTAGGG. Removal of the Sal I site within the plasmid and insertion of an EcoR I site within the DED1 construct was acquired by PCR amplification of the plasmid using primers 5'-CTTATCGATACCGTCTACCTGAGGGGGGGG and 5'-CCCCCCTCGAGGTAGACGGTATCGATAAG for the Sal I deletion and primers 5'-AAACATCAACAGTATGAAATTCGTACTGAGA and 5'-TCTCAGTACGAATTCATCTGTTGATGTTT for the EcoR I insertion. URA3 was PCR amplified from the plasmid pRS316 with primers 5'-AAAGAATTCGTACTGAGAGTGCACCACG and 5'-
GTCGACCTCCTTACGCATCTGTGC. The URA3 PCR product was gel purified, digested with EcoR I and Sal I, and ligated into the modified DED1 construct. The final DED1 construct was referred to as pBSIJKS+-DED1-HA-URA3.

For GST-eIF4G expression in bacteria, eIF4G was PCR amplified from yeast genomic DNA using the primers 5’-AAAGGATCCATGACAGACGAAACTGCTCAC and 5’-AAACTGCAGTTACTCTTCGTCATCACTTTCTC. The PCR product was gel purified, digested with BamH I and Pst I, and ligated into pET41a(+). To obtain eIF4G deletion constructs, PCR amplification of the new plasmid was performed with the primers 5’-TCTACTGTGTCTTTAGCAACCAGA and 5’-TCTGGTGTGCTAAGACACACAGTAGA for eIF4G1-164, 5-AAGGAAAAACTAGGAAGTGCC and 5’-GGCACTTCCTAGTTTTCTTT for eIF4G1-323, 5’-TTCAAGATCTTTAATTCAAGACGTAC and 5’-GTACGTCTTTGAATTAAGGACCTTGAA for eIF4G1-884. To obtain a N-terminal GST tag and a C-terminal Histidine tag for the eIF4G construct, deletion of the Histidine tag within the plasmid was deleted and a C-terminal Histidine tag was added using PCR amplification and the primers 5’PO4-ACCAGAACCACCTAGTTGAACC and 5’PO4-TCCGCGGCTGTCTGGTGCCACG for the deletion and 5’PO4-GTGTGATGATGTTTGATGCTTTGCTGTCATCACTTTCTCC and 5’PO4-TAACTGCGAGCGAGCTCC for the addition. The final N and C terminally tagged eIF4G construct was referred to as pET-Tif N-GST C-HIS.

For co-expression with eIF4G, eIF4E was PCR amplified from yeast genomic DNA using the primers 5’-AAAAACATATGTCGGTTGAAGAAGTTAGCA and 5’-
AAAAACTCGAG TTACAAGGTGATTGATGGTTG. For expression with a C-terminal Histidine tag eIF4E, eIF4E was PCR amplified with the primers 5'-AAAAACATATGTCCGTTGAAGAAGTACCA and 5'-AAAAACTCGAGCAAGGTGATTGATGGTTG. The PCR products were gel purified, digested with Nde I and Xho I, and ligated into pET22b(+).

To obtain a copy of Ded1p on a yeast isogenic plasmid, we digested the pBSIIKS+-DED1-HA plasmid (no URA3 inserted) with Nhe I and Hind III and isolated the ~2.0 kb product by gel purification. Next the pET22b(+) Ded1 plasmid was digested with Nhe I and Hind III and the ~7.0 kb product isolated by gel purification. The two products were ligated together to form a the pET22b(+) His-Ded1-HA construct. Then the N-terminally His tagged and C-terminally HA tagged DED1 gene was PCR amplified using the primers 5’-ATGCACCATCACCACCACCATCACG and 5’-CACCCTAGTTTTGTCTGAAATCA and ligated in to the pYES2.1/V5-His-TOPO vector (Invitrogen) using TA cloning. This plasmid was referred to as pYes-his-Ded1-HA. The NES mutation was made by PCR amplification of the pYes-his-Ded1-HA plasmid using the primers 5'-CGAACAAGTGCAAAATGCAAGCGCCAACGACACACACG and 5'-CGTTGTGCTGTTGGCGCTTTGCACTTGTTCG. The mutated NES plasmid was referred to as pYes-his-Ded1-HA L11A/I13A.

**PROTEIN EXPRESSION AND PURIFICATION**

Ded1p, eIF4G, and eIF4E were expressed in the *E. coli* strain BL21 DE3. The bacteria were grown in LB media (10 g Tryptone, 10 g NaCl, 5 g yeast extract per 1 liter) with the
appropriate selection (Ampicillin 100 µg/L or Kanamycin 25 µg/L) to an OD$_{600}$ of ~0.5-0.7 at 37°C. After induction with 0.5 M IPTG, the bacteria were incubated with vigorous shaking at 28°C (Ded1p) for 22 hrs. or 18°C (eIF4G and eIF4E) for 6 hrs., which precluded the formation of inclusion bodies. Cells were collected by centrifugation at 5000 rpms for 5 min with a GS3 rotor and resuspended in Buffer Y (50 mM NaH$_2$PO$_4$, pH 6.0, 300 mM NaCl, 50% glycerol, 5 mM imidazole, 0.5 % NP$_{40}$, 1 mM PMSF and 1 pellet of Complete™ protease inhibitor cocktail tablets (Roche). Cells were lysed by sonication on ice for 6 cycles of 15 secs. on and 15 secs. off. Cellular debris was pelleted at 15,000 rpms for 30 mins. at 4°C with a SS-34 rotor. The bacterial lysate was then incubated overnight with pre-equilibrated Ni-NTA resin (Qiagen).

The resin was loaded into a 10 cm x 1 cm (length x diameter) column and washed in a sequential manner with 20 mL of 5 mM imidazole and 1 M NaCl buffer Y, 30 mM imidazole buffer Y, and then 60 mM imidazole buffer Y before elution with 250 mM imidazole buffer Y. Fractions containing the desired protein were diluted 1:8 in buffer Z (50 mM Tris-HCl (pH 8.0), 2 mM DTT, 1 mM EDTA, 50 % Glycerol, 0.1 % Triton-X 100) (or for eIF4E, buffer Z2: 50 mM MES pH 5.8, 2 mM DTT, 1 mM EDTA, 50 % Glycerol, 0.1 % Triton-X 100) in order to absorb the protein to pre-equilibrated phosphocellulose (P11, Whatman). After washing with 100 mM NaCl and 200 mM NaCl buffer Z (or Z2), Ded1p was eluted from the phosphocellulose with 300 mM NaCl buffer Z, while eIF4G and eIF4E were eluted with 750 mM NaCl buffer Z (or Z2). Fractions containing eIF4E or Ded1p were aliquoted and stored at -80°C.
Fractions containing eIF4G were diluted 1:8 in buffer C (50 mM Tris pH 7.3, 0.1% Triton-X 100, 50% Glycerol, 1 mM EDTA) and incubated with pre-equilibrated glutathione-sepharose resin overnight. After 2 washes with 150 mM NaCl Buffer C and pre-equilibration, the resin was resuspended in 0.5 mL of Cleavage buffer (20mM Tris pH 8.4, 150 mM NaCl, 2.5mM CaCl₂, 50% Glycerol, 0.1% Triton-X 100). The slurry was incubated with 20 units of Bio-Thrombin (Novagen) for 3hrs at room temperature. The slurry was pelleted and the Bio-Thrombin was removed by passing the supernatant over a streptavidin-agarose spin column (Novagen). The filtrate was aliquoted and stored at -80°C.

Homogeneity (~95%) of all the proteins was assessed by SDS-PAGE and subsequent coomassie staining of the peptides. Concentration of the proteins was determined by the Bradford method and a standard curve produced with known concentrations of bovine serum albumin (BSA, New England Biolabs). U1A containing residues 2 through 117 and TRAP protein were gifts from Dr. Kyoshi Nagai (Cambridge, UK) and Dr. Paul Gollnick (Buffalo, NY) respectively.

**RNA PREPARATION**

RNA oligonucleotides were purchased from DHARMACON (Lafayette) and deprotected according to manufacturer’s protocols. RNA, where applicable, was labeled with $\gamma^{32}\text{P}$-ATP (ICN) using T4 Polynucleotide Kinase (New England BioLabs) and purified on denaturing PAGE. To form RNA duplexes, labeled and complementary unlabeled RNA
were combined in 50 mM KCl, 10 mM MOPS (pH 6.5), 2 mM EDTA, heated to 95°C for 2 min and cooled to room temperature over 2 h. Bipartite complexes were separated from single-stranded RNA by non-denaturing PAGE, visualized by autoradiography, excised, eluted from the gel, and precipitated with ethanol (Jankowsky et al. 2001). RNA complexes were quantified by measuring incorporated $^{32}$P using a scintillation counter.

TRAP RNA was transcribed in vitro from linearized DNA plasmids using T7 polymerase. The 78 nucleotide RNA used for TRAP-RNP remodeling (53 nt TRAP binding site (underlined) and 24 nt single strand overhang 5'-
\[
\text{UGAGGUACCCACACUACACAUAGCCACC}
\]
was prepared by template-directed ligation of two RNA pieces using T4 DNA ligase (M. J. Moore, P. A. Sharp, Science 256, 992-997 (1992)).

**EQUILIBRIUM BINDING FOR U1A AND eIF4G**

Equilibrium binding studies were performed in buffer containing 40 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.01% NP$_{40}$, 10% Glycerol (for eIF4G only), and 2 mM DTT. Radiolabeled RNA (1 nM) in a reaction volume of 10 µL was incubated with increasing concentrations of protein on ice for 10 min, followed by incubation at 19°C for 10 min. Subsequently, 10 µL loading buffer were added to each individual reaction and the solutions were immediately loaded on a non-denaturing PAGE (run at 4°C).
For eIF4G binding to 16 nt RNA, 3µM Ded1p was incubated with 30nM radiolabeled RNA for 2 hrs. at 19°C in buffer containing 40 mM Tris-HCl, pH 8.0, 40mM NaCl, 0.01% NP₄₀, 2.5mM MgCl₂, 2mM DTT, and 2mM ADPNP. The reaction was then diluted 30 fold into 10µl of a solution containing 40 mM Tris-HCl, pH 8.0, 40mM NaCl, 0.01% NP₄₀, 5mM MgCl₂, 10% glycerol, 2mM DTT and eIF4G at concentrations mention in the figure legends. Subsequently, the 10µl was incubated at 19°C for 10min, challenged with a 73 nt RNA (1µM final) for 1 min., and loaded on a non-denaturing PAGE (run at 4°C).

Gels were dried and the bands corresponding to single strand and bound RNA were visualized using a PhosphorImager (Molecular Dynamics). Radioactivity in each band was quantified using the ImageQuant software (Molecular Dynamics). Equilibrium binding constants were calculated according to:

\[
\text{FractionBound} = \frac{K^n}{(K^n + [\text{protein}]^n)}
\]

K is the equilibrium binding constant, n is the Hill-coefficient. Curve fitting was performed using Kaleidagraph (software).

**U1A DISSOCIATION AND DISPLACEMENT**

RNP/RNA remodeling assays were performed as described (40, 96) at room temperature in a buffer containing 40 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.01% (v/v) NP40, and 1 mM DTT with 0.5 nM radiolabeled duplex RNA and U1A, where applicable. U1A–RNA complexes were formed prior to the reaction for 10 min. Spontaneous U1A dissociation
was measured by incubating pre-formed U1A–RNA complexes with a large excess of U1A scavenger RNA (1.25 mM, unlabeled RNA based on U1A binding site in U1 snRNA) (Jankowsky et al. 2001), which prevents rebinding of U1A to the radiolabeled RNA complex once U1A has dissociated from this RNA. Aliquots were removed from the reaction, glycerol (10% v/v final) was added and the aliquots were immediately applied to non-denaturing PAGE (run at 4°C). The fraction of disassembled RNA complexes was determined by quantifying radioactivity in the U1A-bound and free RNA using a Phosphor-Imager (Molecular Dynamics). Dissociation rate constants were determined by plotting the fraction free RNA versus time and fitting the resulting time courses with the integrated rate law for a biphasic first order reaction (sum of two exponentials). Curve fitting was performed using Kaleidagraph software. U1A displacement by Ded1p was measured by performing U1A–RNA complexes as described above. Subsequently, Ded1p (500 nM) was incubated at room temperature with the complex for an additional 5 min. Longer incubation times or higher protein concentrations (U1A, Ded1p) did not alter the results. Remodeling reactions were started by adding 5 mM (final) ATP (where applicable), 5 mM (final) MgCl2, and 1.25 mM (final) U1A scavenger RNA to prevent U1A from rebinding to RNA once it has been displaced. Aliquots were removed at appropriate times and stopped with equal volumes of buffer containing 10 mM EDTA, 1% SDS (v/v), 0.1% bromophenol blue, 0.1% xylene cyanol, and 20% glycerol. Subsequently, aliquots were applied to 15% non-denaturing PAGE (Jankowsky et al. 2001). Gels were dried and the bands corresponding to single strand and duplex RNA were visualized using a PhosphorImager (Molecular Dynamics). Radioactivity in each band was quantified using the ImageQuant software (Molecular Dynamics).
In the presence of U1A, single-stranded RNA corresponds to the amount of displaced U1A (40). We specifically confirmed that Ded1p did not displace U1A without separating the RNA strands (data not shown). Rates for strand separation were determined by plotting the fraction single-stranded RNA versus reaction time and fitting the resulting time courses with the integrated rate law for a homogenous first order reaction (without U1A) or with the integrated rate law for a biphasic first order reaction (with U1A bound).

**THERMAL MELTING CURVES**

Melting temperatures of wildtype and altered duplexes (cf. Chapter 2, Figure 2.10) were determined in reaction buffer containing 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM MgCl₂, 2 mM DTT, 1 unit/µL RNasin, 0.01% NP40. The duplexes at 0.5 nM were incubated at indicated temperatures ranging from 24 to 95 °C in a temperature calibrated PCR machine for 5 min. Then reactions transferred on ice and buffer containing 1% SDS, 50 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, 20% glycerol was added. Subsequently, aliquots were applied to a 15% non-denaturing polyacrylamide gel, duplex and single-stranded RNAs were resolved, and the fraction of single stranded RNA was determined and plotted vs. incubation temperature.

**MICROCOCCAL FOOTPRINTING**

U1A footprinting studies were conducted in buffer containing 40 mM CaCl₂, 50 mM NaCl, 40 mM Tris, pH 8.0, 2 mM DTT, and 0.01% NP₄₀ in a reaction volume of 150µl. Radiolabeled RNA duplex (5nM) was incubated with 125 nM U1A on ice for 10 min.
After a 5 min. incubation at 37°C, the reaction was started by adding 0.01U of micrococcal nuclease. The reactions continued for 6 min, were placed on ice, and stopped by the addition of 15µl of 38.8mM EGTA. The RNA was precipitated by ethanol and loaded onto a denaturing PAGE. Gels were dried and the RNA was visualized using a PhosphorImager (Molecular Dynamics).

**TRAP DISSOCIATION AND DISPLACEMENT**

TRAP-RNP remodeling reactions were performed at room temperature in a buffer containing 40 mM Tris-HCl pH 8.0, 0.01% (v/v) Nonidet P40, 0.5 nM radiolabeled RNA, and 50 mM NaCl. The following concentrations of applicable components were used in reactions for Ded1p: 3 mM ATP, 3 mM MgCl₂, 500 nM Ded1p, 20 nM TRAP (monomer), 600 nM TRAP RNA scavenger DNA, 0.5 nM RNA control duplex, 10 µM tryptophan. Higher tryptophan concentrations did not significantly change the remodeling rate, lower tryptophan concentrations significantly increased the rate of spontaneous TRAP dissociation.

TRAP-RNA complexes were formed prior to the reaction for 10 minutes. Subsequently, Ded1p was added and incubation was continued for at least 5 minutes. Longer incubation times did not change the observed results. Remodeling reactions were started by adding a mixture of ATP and RNA scavenger. The latter consists of DNA oligonucleotides that hybridize to the TRAP binding site and thus prevent re-binding of TRAP to its cognate RNA once it has been displaced. Aliquots were removed at appropriate times and reactions were stopped by EDTA (5 mM final) and Ded1p scavenger RNA, that prevents
Ded1p from binding to the RNA from which TRAP has been displaced. The aliquots were applied to 8% non-denaturing PAGE (1.5 mm thick, run at 10 V/cm at 4°C). Gels were dried and bands corresponding to free and complexed RNA were visualized using a PhosphorImager (Molecular Dynamics). Radioactivity in the respective bands was quantified using the ImageQuant software (Molecular Dynamics). The fraction of displaced TRAP (Fraction \([P]\)) was calculated from the amount of radioactivity in the TRAP species ([TRAP]) and the amount of radioactivity in the free RNA ([RNA]) according to

\[
Fraction[P] = \frac{[RNA]}{([RNA] + [TRAP])}
\]

Dissociation and displacement rate constants were determined from plots of Fraction \([P]\) vs. time by least square fitting to the integrated rate law of a first order reaction using Kaleidagraph (Synergy software).

**YEAST TRANSFORMATION**

Yeast grown at 30°C in 50 mL of YPD (1% yeast extract, 2% Peptone, 2% glucose) to an OD\(_{600}\) of ~1 were collected by centrifugation at 4200 xg for 10 min. at 4°C. After washing twice with 10 mL of enzyme grade H\(_2\)O and once with 10 mL of TE/LiAc buffer (0.1 mM LiAc, 10mM Tris pH 7.5, 1mM EDTA), the yeast were incubated in 500 µL of TE/LiAc Buffer on ice for 1 hr. A 50 µL aliquot of the yeast was then incubated with 20 µg of carrier salmon sperm DNA, 300 µL of PEG solution (40% w/v PEG-4000, 100 mM LiAc, 10mM Tris pH 7.5, 1mM EDTA), and either 100 ng of DNA for an isogenic plasmid or 1 µg of DNA for homologous recombination at 30°C for 30 min. with shaking. After an shifting the yeast to 42°C for 20 min., the cells were harvested by
centrifugation, washed, and plated onto synthetic dropout plates lacking uracil (0.67% nitrogen base without amino acids, 1% (NH₄)₂SO₄, 2% Glucose, 1 µg/mL Adenine, 1µg/mL Leucine, 0.5µg/mL Histidine, and 2% Agar).

**YEAST EXTRACTS**

Yeast were grown at 30°C in YPD to an OD₆₀₀ ~1 and collected by centrifugation at 4200 xg for 10 min. at 4°C. After washing twice with 10 mL of enzyme grade H₂O, yeast were resuspended in lysis buffer (50 mM KCl, 5 mM MgCl₂, 10mM HEPES, pH 7.4, 1% NP₄₀, 1mM DTT, 1mM PMSF, 1 µg/mL pepstatin, 1 µg/mL Aprotinin, 10 µg/mL leupeptin, 0.14M ß-mercaptoethanol). Glass beads (0.5 mL) were added to the yeast. The cells were then snap froze in liquid nitrogen for 2.5 min. and thawed at 50°C for 2.5 min. The cells and beads were vortexed for 1 min and immediately placed on ice for 1 min. Another round of freeze-thawing and bead lysis occurred before the cellular debris was removed by centrifugation at 4200 xg for 5 min. at 4°C. Extracts from different strains were normalized by dilution with lysis buffer using the concentrations obtained from the Bradford Assay.

**CO-IMMUNOPRECIPITATIONS**

Lysates from the yeast strains BY4741 and BY4741.HA were incubated with 5 µl of pre-equilibrated EZ-view α-HA beads (Sigma) overnight at 4°C. The beads were pelleted and washed three times with RIPA buffer (50 mM Tris, pH 7.4, 1% NP₄₀, 0.25% sodium deoxycholate, 50mM KCl, 1mM EDTA, 1mM PMSF, 0.1% SDS, 1 µg/mL pepstatin, 1 µg/mL Aprotinin, 10 µg/mL leupeptin, 0.14M ß-mercaptoethanol). For the majority of
co-immunoprecipitations, the beads were placed in 25 µl of laemmlie buffer and the proteins resolved by SDS-PAGE. For the identification of potential helicase cofactors, the beads were incubated with 1 µg of HA peptide (Sigma) in 0.5 mL of RIPA buffer at room temperature for 1 hr with vigorous shaking. The protein from the eluate was precipitated by adding trichloroacetic acid to a final concentration of 10% and incubating the eluate at 4°C overnight. The precipitate was pelleted at 16.1 xg for 30 min at 4°C, washed with Acetone/Tris buffer (5 parts acetone, 1 part 1 M Tris, pH 8.0), and resuspended in 25 µL laemmlie buffer. Proteins were resolved by SDS-PAGE.

MASS SPECTROMETRY

The mass spectrometry was conducted by Dr. Michael Kinter and the Lerner Research Institute Mass Spectrometry Laboratory for Protein Sequencing at the Cleveland Clinic Foundation. Eleven bands were cut from a Coomassie blue stained 1D gel taking care to minimize the amount of polyacrlylamide (dated 7/16/06-07-19/06 of proteins after series of purifications). The bands were washed, destained in 50% ethanol/5% acetic acid and dehydrated in acetonitrile. In-gel proteolytic digestion using trypsin was accomplished by adding 5 µL 20 ng/µL trypsin in 50 mM ammonium bicarbonate and incubating overnight at room temperature. The peptides that were formed were extracted from the polyacrlylamide in two aliquots of 30µL 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to <10 µL in Speedvac and then resuspended in 1% acetic acid to make up a final volume of 30 µL for LC-MS analysis.
The LC-MS system was a Finnigan LCQ ion trap mass spectrometer system. The HPLC column was a self-packed 11 cm x 75 µm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. Two µL volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of 0.3 µL/min were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source is operated at 2.5 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 2500 collisionally induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude. Please note that not all CID spectra are derived from peptides.

The data were analyzed by using all CID spectra collected in the experiment to search the NCBI non-redundant database with the search program Mascot using fungi taxonomy filter. All matching spectra were verified by manual interpretation. The interpretation process was aided by additional searches using the programs Sequest and Blast as needed.

**GST-PULLDOWNS**

GST constructs in bacterial lysates or purified GST or GST-eIF4G (160nM) was incubated with 5 µl of glutathione-sepharose resin for 4 hrs. at 4°C (GE Healthcare). After 3 washes with RIPA buffer, the resin was incubated with purified Ded1p (80nM)
and 0.5% (w/v) BSA (Roche) in buffer containing 40mM Tris, pH 8.0, 0.01% NP₄₀, 2mM DTT, 50mM NaCl, 14% glycerol, and 5mM MgCl₂ overnight at 4°C. The addition of ATP analogs (2mM) and MgCl₂ (2mM) were added when mentioned. The resin was washed 3 more times with RIPA buffer. The bound proteins were eluted from the resin with 25 µl of Laemmli buffer and heating to 95°C for 5 mins. Bands were resolved on 4-15% SDS-PAGE gradient gels.

WESTERN BLOT ANALYSIS

SDS-PAGE gels were transferred to pre-wet Immobilon-P paper in buffer containing 25mM Tris, 20% methanol, 192mM Glycine, 0.01% SDS at 100V for 1hr at 4°C. The Immobilon-P was then washed with PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4 mM KH₂PO₄) and incubated with blocking buffer (5% w/v milk, PBS) for 1 hr. The membrane was washed twice with PBST (PBS, 0.1% Tween-20), and once with PBS. Incubation with a primary antibody in 5% milk PBS (0.5 mg/ml BSA in PBS for α-eIF4G) occurred for 1hrs. Incubation duration depended on the dilution of the antibody which was generally 1:12,500 for α-Ded1p, 1:3000 for α-eIF4G, 1:15,000 for α-S tag-HRP, 1:1000 for α-Pfkp. Again the Immobilon-P was was washed twice with PBST (PBS, 0.1% Tween-20), and once with PBS. If secondary antibody was needed, the membrane was incubated with a secondary antibody in 5% milk PBS for 1 hr. Finally the blot was washed thrice with PBST. The SuperSignal West Pico Chemiluminescent Substrate (pierce) was prepared according to manufactures protocols and applied to the blot for 1 min. before exposing to autoradiography film.
ATPASE ASSAY

ATPase assays were conducted as described in (106). Briefly, 2 mM phosphoenolpyruvate (PEP), 0.6 mM NADH, 0.18 u pyruvate kinase (Roche), 0.54 u lactose dehydrogenase (LDH, Roche), and eIF4G at increasing concentration were incubated at 19ºC for 5 min. in buffer containing 40 mM Tris, pH 8.0, 0.01% NP40, 2.5 mM MgCl2, 2 mM ATP, and 2.5 µM of a 16 bp RNA duplex with a 24 nt ssRNA region appended onto the duplexes 3’ end. Pre-equilibrated Ded1p (100nM) was added to start the reactions and NADH absorbance was monitored at 340 nm for 5 min at 30 sec. intervals and 19ºC using a Beckman DU800. The rate of change in NADH absorbance was obtained from the slope of the line from the plot of NADH absorbance against time. Rates of ATP hydrolysis were obtained by multiplying the NADH extinction coefficient at 340 nm (6300 M⁻¹ cm⁻¹) and the slope.

TRYPTOPHAN FLUORESCENCE

Fluorescence studies were conducted in buffer containing 40 mM Tris, pH 8.0, 0.1 % NP40, 2 mM DTT, and 0.5 mM MgCl2. Increasing concentrations of the mRNA cap analog, ARCA, was incubated with 0.5 µM eIF4E at room temperature for 5 min. The reactions were then excited at 295 nm and Tryptophan fluorescence was measured between 315 and 400 nm using the Fluoromax spectrophotometer. The data was extracted to a Microsoft Excel file and background fluorescence of buffer and ARCA were subtracted from the eIF4E fluorescence.
DATA ANALYSIS FOR UNWINDING AND ANNEALING REACTIONS

Data analysis for unwinding and annealing reactions were conducted as described in (64).
REFERENCES


