MOLECULAR MECHANISM OF THE TRAMP COMPLEX

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
Dedication

“But lately it seems to me that dedicating a book is like the fine rhetoric about offering one's life to one's country, or handing the reins of the government back to the people. This is but the vain and empty juggling of language. Despite all the talk about handing it over, the book remains like the flying knife of the magician—released without ever leaving the hand. And when he dedicates his work in whatever manner he chooses, the work is still the author's own. Since my book is a mere trifle, it does not call for such ingenious disingenuousness. I therefore have not bothered myself about the dedication.”

Quoted from the preface of Fortress Beseiged by Qian Zhongshu (Ch’ien Chung-shu). Translated by Jeanne Kelly and Nathan K. Mao.
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List of Abbreviations

ABC transporter, ATP-binding cassette transporter
ADP, adenosine diphosphate
ADP-AlF$_4$, ADP-aluminum fluoride
ADP-BeF$_x$, ADP-beryllium fluoride
ADPNP, adenylyl imidodiphosphonate
ARC complex, Argonaute siRNA chaperone complex
ATP, adenosine triphosphate
ATPase, adenosine triphosphatase
bp, base pair
Btz, Barentsz
CARD, Caspase activation and recruitment domains
CoTC, cotranscriptional cleavage
C-terminal/terminus, carboxyl terminal/terminus
CUTs, cryptic unstable transcripts
DNA, deoxyribonucleic acid
dsRBD, double-stranded RNA binding domain
dsRNA, double-stranded RNA
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
EJC, exon junction complex
EMT, epithelial-mesenchymal transition
5’ ETS, 5’ external transcribed spacer
FN3 domain, fibronectin type III domain
FRET, Förster resonance energy transfer
GTP, guanosine triphosphate
HCV, hepatitis C virus
IGS, intergenic spacer
IP6, inositol hexakisphosphate
ITP, inosine triphosphate
KOW domain, Kyprides, Ouzounis, Woese domain
m1A methyltransferase, 1-methyladenosine methyltransferase
mRNA, messenger RNA
mt, mutant
NES, nuclear export signal
NMD, nonsense-mediated decay
nt, nucleotide
N-terminal/terminus, amino terminal/terminus
NTP, nucleotide triphosphate
OB-fold, oligonucleotide/oligosaccharide binding-fold
PAGE, polyacrylamide gel electrophoresis
PAP, poly(A) polymerase
PAZ domain, Piwi, Argonaut, Zwille domain
P-body, processing body
P-Loop, phosphate-binding loop
Pol I, II, III, RNA polymerase I, II, III
poly(A), polyadenylic acid
PTC, peptidyl transferase center
3’ RACE, 3’ rapid amplification of cDNA ends
RITS complex, RNA-induced transcriptional silencing complex
RNA, ribonucleic acid
RNP, ribonucleoprotein
rRNA, ribosomal RNA
RRM, RNA recognition motif
SDS, sodium dodecyl sulfate
ssRNA, single-stranded RNA
snRNA, small nuclear RNA
snoRNA, small nucleolar RNA
SF, super family
TAP, tandem affinity purification
TRAMP, Trf4/Air2/Mtr4 polyadenylation
Tris, tris(hydroxymethyl)aminomethane
tRNA, transfer RNA
tRNA\text{\textsuperscript{\text{\text{Met}}}}, tRNA initiator methionine
UTP, uridine triphosphate
WH domain, winged-helix domain
wt, wildtype
The TRAMP complex (Trf4/Air2/Mtr4 polyadenylation) is required for quality control and 3’ maturation of many RNAs in the nucleus. The TRAMP complex consists of three units, the poly(A) polymerase Trf4/5p, the zinc-knuckle protein Air1/2p, and the Ski2-like RNA helicase Mtr4p. Using recombinant *Saccharomyces cerevisiae* TRAMP complex, I found that the RNA helicase Mtr4p modulates activities of the polymerase Trf4p and restricts the length of oligo(A) appended after addition of ~4 nt. The modulation does not require duplex unwinding but relies on sensing of oligo(A) length by Mtr4p. Trf4p, in turn, stimulates the unwinding activity of Mtr4p independent of polyadenylation. Polyadenylation of duplex RNAs by Trf4p creates a landing site for Mtr4p to start unwinding, the length of oligo(A) required coincides with restriction of adenylation by Mtr4p. The functional cross-talk between Trf4p and Mtr4p in the TRAMP complex promotes addition of a minimum oligo(A) tail (~4 nt) and may be critical for sorting of polyadenylated RNAs in vivo and for subsequent specific target processing by the nuclear exosome.

The *S. cerevisiae* DEAD-box protein Ded1p unwinds RNA duplexes by local strand separation instead of translocation. In this study, I established that distinct units of Ded1p exist on single-stranded and duplex regions; Ded1p unit(s) bound to single-stranded regions facilitates loading of additional unit(s) of Ded1p to duplex regions for unwinding. I further
analyzed Ded1p binding to single-stranded RNA in the presence of analogs for different states of ATP hydrolysis. Each Ded1p monomer binds ~10 nt ssRNA in the presence of the non-hydrolyzable analog ADPNP. The ground state analog ADP-BeF₄ and the transition state analog ADP-AlF₄ lead to binding of more Ded1p molecules to the same length of RNA, probably through Ded1p oligomerization. Such ATP analog-induced association of additional Ded1p molecules likely takes place during duplex unwinding.
Chapter 1

General introduction

1.1 Introduction to RNA helicases

RNA helicases are found in all domains of life (Anantharaman et al., 2002). These proteins bind or remodel RNA or RNA-protein complexes in an ATP-dependent fashion (Pyle, 2008; Jankowsky, 2011). RNA helicases are involved in many aspects of RNA metabolism in the cell, such as mRNA splicing, ribosome biogenesis, mRNA translation, etc. (Tanner and Linder, 2002).

Since the identification of RNA helicases in the 1980s (Grifo et al., 1984), a large body of knowledge has been generated for these enzymes. Crystal structures are available for a number of RNA helicases, some with both ATP analog and RNA bound (Section 1.3). Biochemical characterization of RNA helicases has provided valuable information on the various activities of these proteins (Sections 1.4, 1.5). Cellular roles of RNA helicases have also been studied with great enthusiasm (Section 1.5). Several central questions, however, remain to be elucidated. These include the mechanism of helicase action (Section 1.4), the activity of RNA helicases on cellular targets (Section 1.5), and the activity of RNA helicases with physiological protein partners (Section 1.6). This thesis presents mechanistic studies of two RNA helicases, Ded1p and Mtr4p, and a protein complex containing an RNA helicase, the TRAMP complex (Trf4/Air2/Mtr4 polyadenylation). The work also hints on the cellular function of Mtr4p in the TRAMP complex.
1.2 Classification of helicases

Helicases belong to the class of P-loop NTPases which also include kinesin, myosin, G proteins (guanine nucleotide binding proteins), ABC transporters (ATP-binding cassette), AAA⁺ proteins (ATPases associated with diverse cellular activities), etc. (Walker et al., 1982; Saraste et al., 1990). Based on sequence homology, all helicases are classified into six superfamilies, SF1-6 (Gorbalenya and Koonin, 1993; Singleton et al., 2007). SF1 and SF2 helicases contain two globular domains named after the *Escherichia coli* protein RecA (Caruthers and McKay, 2002). All eukaryotic RNA helicases belong to SF1 and SF2 (Figure 1.1). SF3-6 helicases exist as hexameric rings of single RecA-like domains (Singleton et al., 2007), and are only found in bacteria and viruses (Jankowsky, 2011).

SF1 and SF2 helicases are further divided into families based on sequence and structural comparisons (Fairman-Williams et al., 2010) (Figures 1.1, 1.2). Several families contain both RNA and DNA helicases, and some helicases work on both DNA and RNA (Fairman-Williams et al., 2010). The majority of eukaryotic RNA helicases fall into SF2 and only the Upf1-like family in SF1 contains RNA helicases (Fairman-Williams et al., 2010). SF2 families with RNA helicases include the DEAD-box, DEAH/RHA, Ski2-like, RIG-I-like and NS3/NPH-II families (Fairman-Williams et al., 2010) (Figure 1.1).
1.3 Sequence and structural features of SF2 RNA helicases

1.3.1 Conserved sequence motifs in the helicase core

The helicase core of an SF2 helicase is formed by two RecA-like domains. Each of the RecA-like domains contains a parallel β-sheet cradled by α-helices on either side (Figures 1.2, 1.3). Thirteen characteristic sequence motifs have been identified in SF2 helicases, with roles involving ATP binding/hydrolysis, RNA binding and the coupling between the two (Fairman-Williams et al., 2010) (Figure 1.2).
Figure 1.2 Sequence and structural organization of the helicase core of SF2 proteins (Fairman-Williams et al., 2010).

(A) Sequence organization of the helicase core in SF2. Characteristic sequence motifs are colored according to their predominant biochemical function: red, ATP binding and hydrolysis; yellow, coordination between nucleic acid and NTP binding sites; blue, nucleic acid binding. Green circled asterisks mark insertions of additional domains. The lengths of the blocks and the distance between the conserved domains are not to scale. Characteristic motifs were identified from the alignment of all SF2 proteins from human, S. cerevisiae, E. coli and selected viruses. Considering numbering schemes already in use (Jankowsky and Fairman, 2007), motifs were numbered consecutively. The Q-motif is equivalent to motif 0 in RecQ proteins. Motif IVa in SF2 proteins is frequently marked QxxR, motif Ic often TPGR. The asterisk on motif Ib indicates that in some proteins this motif is replaced by an additional domain.

(B) Sequence conservation within the characteristic helicase motifs. The height of the amino acids reflects the level of conservation at a given position, taller letters indicate higher conservation. The universally conserved E in motif II corresponds to 4 bits. Coloring marks the chemical properties of a given amino acid position: green and purple—polar, blue—basic, red—acidic, and black—hydrophobic. Sequence logos were created from the alignment of SF2 proteins according to (Crooks et al., 2004). Circles under the letters are for visual guidance.

(C) Position of the characteristic motifs in the RecA-like folds of the helicase core domains. The β-strands are indicated by arrows, α-helices by cylinders. The β-strands of the first RecA-like domain are numbered according to their position in the primary structure. The position of the characteristic motifs is indicated by numbered circles, colored as in panel A. The position of inserted domains is marked by green circled asterisks, as in panel A. Blue coloring of the rightmost β-strand and α-helix indicates the absence of this part in several SF2 protein families.

Adapted with permission from Elsevier (Fairman-Williams et al., 2010).
The Q-motif confers specificity for the adenine base through hydrogen bonding and stacking with the base. Motif I and motif II correspond to Walker A and Walker B motifs required for NTP binding and hydrolysis (Walker et al., 1982). Walker A (motif I) is also known as the P-loop (phosphate-binding loop). It contacts the β-phosphate as well as the magnesium ion bound between the β- and γ-phosphates. Motif II (walker B) coordinates the magnesium ion and the catalytic water molecule. Motif VI interacts with the γ-phosphate and plays a role in stabilizing the transition state of ATP hydrolysis. The second arginine in motif VI is referred to as the arginine finger, and has been found to have similar functions in other P-loop proteins such as G-proteins (or their activators) (Tesmer et al., 1997; Scheffzek et al. 1997; Rittinger et al., 1997) and AAA⁺ proteins (Zhang et al., 2002).

Motifs Ia, Ib, Ic, IVa, IV, V and Vb are involved in RNA binding. Most of the contacts to the RNA are electrostatic interactions with the phosphates. Many of the 2'-hydroxyl groups are recognized as well by the RNA-specific SF2 helicases.

Motifs III and Va function in the coupling between ATP binding/hydrolysis and RNA binding. Motif III is often referred to as the “sensor” motif because of its role in sensing γ-phosphate release during ATP hydrolysis. Mutations in motifs III and Va usually maintain ATP binding/hydrolysis activity but show markedly impaired unwinding activity (e.g. Tanaka and Schwer, 2006; Banroques et al., 2010).

With the conserved motifs, ATP is bound mostly by the N-terminal RecA-like domain but is held by motif VI in the C-terminal RecA-like domain (Figures 1.2, 1.3). Different conformations of the two RecA-like domains in the presence and absence of ADP or ATP analogs have been observed in crystal structures of DEAD-box proteins (Caruthers et al., 2000; Shi et al., 2004; Bono et al., 2006), and in other families (Luo et al., 2008; Gu and
In all cases, the two RecA-like domains open in the absence of nucleotide and close upon binding of ATP analogs.

RNA binds on the side opposite of the ATP-binding site, to a largely basic surface formed by both RecA-like domains (Figure 1.3). Perhaps expected for this arrangement of ATP and RNA binding sites, ATP binding/hydrolysis induced different conformations of RNA binding. Crystal structures of SF1 and SF2 helicases indicate that the distance between two highly conserved threonines (occasionally substituted by serine) from motif Ic and V, respectively (Figure 1.2B), decreases from 3 nt to 2 nt upon ATP-analog binding (Myong et al., 2007). The observation was used to explain the translocation of the Hepatitis C virus NS3 helicase at 1 nt per ATP (Myong et al., 2007).

![Figure 1.3](image)

**Figure 1.3 Structure of the DEAD-box protein Vasa with ADPNP and ssRNA bound (Sengoku et al., 2006).** The two RecA domains are in a “closed” conformation. The ATP analog ADPNP is colored magenta, the ssRNA (7 nt poly(U)) is colored orange. Conserved sequence motifs are colored as in Figure 1.2.
1.3.2 N- and C-terminal domains

Most SF2 helicases contain N- and C-terminal sequences in addition to the helicase core. Some of these sequences fold into defined domain structures that are important for RNA binding or other specific functions (Figure 1.4). Some degree of structural conservation is observed within families (e.g. RIG-I, Mda5 and LGP2 in RIG-I-like family (Yoneyama and Fujita, 2010)) and between families (e.g. Hel308 and Brr2p in Ski2-like family and Prp43p in DEAH/RHA family (Buttner et al., 2007; Pena et al., 2009; Zhang et al., 2009; He et al., 2010)) (Figure 1.4).

Figure 1.4 Domain organization of SF2 RNA helicase families (Fairman-Williams et al., 2010). Domains are not to scale.
(A) C-termini and N-termini of DEAD-box proteins include RRMs, Zn-fingers, tudor domains and others (Linder, 2006).
(B) The family-typical domain inserted between the helicase domains is shown in grey. RIG-I-like proteins vary in their terminal domains (Yoneyama and Fujita, 2008). Prominent RIG-I-like proteins are shown, Mph1p/FancM-related proteins are not shown (CARD: caspase recruitment domain, RD: regulatory domain, a Zn-binding domain (Cui et al., 2008), PAZ: PIWI, Argonaute, Zwille, dsRBD: double-stranded RNA binding domain).
(C) Domain organization of Hel308 (Buttner et al., 2007). The organization of the C-terminal domains is conserved in Brr2 (Zhang et al., 2009; Pena et al., 2009) (WH: winged helix, H1: helical 1, H2: helical 2, FN3: fibronectin 3).
(D) DEAH/RHA proteins have varying N-terminal domains, but show a very high degree of conservation in their C-termini, especially among the spliceosomal DEAH proteins. It is possible that DEAH proteins and perhaps even most DEAH/RHA proteins show a conserved domain organization of their C-termini. Shown is the domain organization of Prp43p (He et al., 2010). The domain organization of the C-terminus, with the exception of the OB-fold domain, resembles that of Ski2-like proteins (Buttner et al., 2007; Zhang et al., 2009; Pena et al., 2009) (WH*: degenerated winged helix, Ratchet corresponds to H1 in the Ski2-like proteins).
(E) NS3/NPH-II proteins have pronounced C-terminal and N-terminal domains, but with the exception of the shown helical C-terminus of NS3 proteins from *Flaviviridae* (Kim *et al.*, 1998), no further information about these domains is available. Modified with permission from Elsevier (Fairman-Williams *et al.*, 2010).

### 1.3.3 β-hairpin structure

![Diagram of β-hairpin structure](image)

**Figure 1.5** β-hairpin structure in SF2 RNA helicases (Fairman-Williams *et al.*, 2010).

(A) Position of the β-hairpin relative to characteristic helicase motifs. Motifs colored as in Figure 1.1. The hairpin is represented by the asterisk in green circle.

(B) β-hairpin in the DEAH/RHA family protein Prp43p (*S. cerevisiae*) (He *et al.*, 2010). The 1st RecA domain is in blue, the 2nd RecA domain is in pink and accessory/inserted domains are in light beige. The hairpin is in green.

(C) β-hairpin in the Ski2-like protein Hel308 (*Archaeoglobus fulgidus*) (Buttner *et al.*, 2007). Colored as in panel B. The nucleic acid (DNA in this case) is in orange.

(D) β-hairpin in the NS3/NPH-II family protein HCV NS3 (*Hepatitis C virus*) (Yao *et al.*, 1999). Colored as in panel C.

(E) Lack of β-hairpin in the DEAD-box protein Vasa (*Drosophila melanogaster*) (Sengoku 2006). Proteins of the RIG-I-like family do not seem to have the hairpin, either (Fairman-Williams *et al.*, 2010).

Modified with permission from Elsevier (Fairman-Williams *et al.*, 2010).

A β-hairpin structure in the C-terminal RecA-like domain was first identified in the Ski2-like helicase Hel308 from *Archaeoglobus fulgidus*, and implicated in duplex unwinding (Buttner *et al.*, 2007) (Figure 1.5). The domain organization of Hel308 is similar to Prp43p, except that an HLH (helix-loop-helix) domain replaces the OB-fold (oligonucleotide/
In the structure of Hel308, the DNA duplex was partially opened by 2 bp, due to the presence of the β-hairpin at the junction between duplex and single-stranded regions. Several hydrogen bonds to both strands of the proximal end of the duplex region were observed, from the C-terminal RecA-like domain and the ratchet domain. The 3’ single-stranded overhang region was held in place by the RecA-like domains, the additional WH (winged helix) and ratchet domains plus an HLH domain. Similar hairpin structures were seen in DEAH/RHA and NS3/NPH-II families, but not in DEAD-box or RIG-I-like families (Fairman-Williams et al., 2010) (Figure 1.5). No SF2 RNA helicase has been crystalized with an RNA duplex bound. However, the position of the β-hairpin is conserved, and the structure likely plays a role in duplex unwinding (e.g. Gu and Rice, 2009; He et al., 2010).

1.4 Unwinding mechanism of SF2 RNA helicases

In vitro, RNA helicases have been shown to unwind RNA secondary structures (Jankowsky, 2011), remove proteins from RNA (Jankowsky et al., 2001; Fairman et al., 2004; Bowers et al., 2005), promote strand annealing (Rossler et al., 2001; Chamot et al., 2005; Yang et al., 2005; Halls et al., 2007), and catalyze RNA structural conversion (Yang et al., 2007b; Bhaskaran and Russel, 2007). Although the main function of a given RNA helicase in the cell may not be unwinding per se, to understand the unwinding mechanism provides a basis for interpretation of other activities, which probably also involve the ATP-dependent or ATP-modulated ability to bind RNA substrates (Jankowsky, 2011).
At least two types of duplex unwinding mechanism exist for SF2 RNA helicases: canonical translocation-based unwinding and local strand separation. The canonical translocation-based mechanism is represented by two proteins in the NS3/NPH-II family: the *Hepatitis C virus* NS3 and the *Vaccinia virus* NPH-II. Both NS3 and NPH-II unwind RNA duplexes in a 3’->5’ directionality (Dumont et al., 2006; Gross and Shuman, 1996a). Following initiation on the single-stranded region, translocation is limited to the strand containing the 3’ unpaired region (loading/tracking strand) and unaffected by defects in the other strand (Frick, 2007; Gross and Shuman, 1996a; Jankowsky et al., 2000; Kaowaka et al., 2004). Directional translocation through the duplex region is tightly coupled to the ATP binding/hydrolysis cycle. For NS3, it has been shown that the helicase translocates 1 nt per ATP turnover (Myong et al., 2007; Gu and Rice, 2010), similar to SF1 DNA helicases such as PcrA and UvrD (Velankar et al., 1999; Lee and Yang, 2006).

Proteins of the DEAH/RHA, Ski2-like and RIG-I-like families also prefer 3’ single-stranded overhangs for duplex unwinding (Jankowsky, 2011). Polar unwinding (3’ vs. 5’ overhangs), however, does not necessarily indicate translocation. The exact mechanism of unwinding remains to be elucidated for these families. Chapter 4 of this thesis includes an analysis of the Ski2-like helicase Mtr4p from *Saccharomyces cerevisiae*.

DEAD-box helicases unwind duplexes by local strand separation, an unwinding mode not based on translocation (Yang and Jankowsky, 2006; Tijerina et al., 2006; Yang et al., 2007a). Single-stranded extensions regardless of orientation facilitate loading of the enzyme directly onto duplexes regions (Yang and Jankowsky, 2006). The enzyme then locally opens a small number of base pairs either from the ends or from internal regions of the duplex (Yang et al., 2007a). ATP binding to a DEAD-box protein is sufficient for duplex
unwinding; ATP hydrolysis is only necessary for enzyme release from the RNA (Chen et al., 2008; Liu et al., 2008). This unwinding mechanism will be discussed in detail in Chapter 6 and is further investigated in Chapters 7-9, using the *S. cerevisiae* DEAD-box protein Ded1p.

### 1.5 Activities of SF2 RNA helicases with relationship to cellular functions

#### 1.5.1 Diverse cellular functions of SF2 RNA helicases

SF2 RNA helicases are found in virtually all aspects of cellular RNA metabolism (Figure 1.6). Some RNA helicases function in a single pathway, such as Dbp8p in ribosome biogenesis (Daugeron et al., 2001). Others have been implicated in multiple processes, such as Ded1p in ribosome biogenesis (Thuillier et al., 1995; Schafer et al., 2003), mRNA splicing (Jamieson et al., 1991), mRNA storage (Beckham et al., 2008), and translation (Chuang et al., 1997; de la Cruz et al., 1997). Some cellular processes only involve one particular family of SF2 helicases, such as cytosolic pathogen recognition by RIG-I-like proteins (Yoneyama and Fujita, 2010), mRNA export involving DEAD-box proteins (Stewart, 2010) (Figure 1.6). Other pathways are accomplished by RNA helicases from several families, such as pre-mRNA splicing and mRNA translation, which utilize DEAD-box, DEAH/RHA and Ski2-like proteins (e.g. Staley and Woolford, Jr., 2009; Linder, 2003; Seafoss et al., 2001) (Figure 1.6).

The link between biochemical activities and *in vivo* functions has been largely unclear. It is also not known why a particular helicase is suited for a certain function. Sections 1.5.2-1.5.5 discuss biochemical activities of SF2 RNA helicases implicated in specific cellular functions with known RNA targets. Chapter 3 of my thesis adds to the list
and reports a novel activity of an RNA helicase, tuning of another enzyme in response to features in the RNA. The activity was used to explain sorting of poly(A)$^+$ RNAs in the nucleus.

1.5.2 RNA duplex unwinding

The *Escherichia coli* DEAD-box protein DbpA displays robust ATPase activity only in the presence of a stem-loop structure (hairpin 92) from the peptidyl transferase center (PTC) of 23 rRNA (Fuller-Pace *et al.*, 1993). As was proposed for DbpA and its homolog YxiN from *Bacillus subtilis*, recruitment to the stem-loop structure by a C-terminal additional domain is thought to result in unwinding of an adjacent duplex by the helicase core (Diges and Uhlenbeck, 2001; Karginov *et al.*, 2005). Duplex unwinding by DbpA has thus been implicated in the assembly of the large ribosomal unit (Sharpe *et al.*, 2009).

Spliceosomal SF2 helicases have been assigned to distinct steps of the splicing reaction, and models for the conformational rearrangements in snRNAs and pre-mRNA exist for each step (Staley and Woolford, Jr., 2009). For example, the *S. cerevisiae* DEAH/RHA protein Prp5p has been proposed to activate U2 snRNA by disrupting stem IIc and forming instead stem IIa (Perriman and Ares, Jr., 2007). Another DEAH/RHA protein Prp16p is implicated in a second disruption of stem IIc to form stem IIa after the 1$^{st}$ catalytic step of splicing (Perriman and Ares, Jr., 2007). While stem IIc is the 3’ most duplex in U2 alone (Prp5p) and in U2 paired with U6 (Prp16p), it is not known why a 3’->5’ unwinding reaction will not further disassemble the RNAs. A possible mechanism involves sequestration of non-target duplex regions by other proteins (see an example below). It is also unclear whether formation of stem IIa is promoted by an annealing activity.
Figure 1.6 Cellular roles of eukaryotic RNA helicases (Jankowsky, 2011).
Selected basic processes of eukaryotic RNA metabolism are represented by the white circles, as indicated by the callouts (NMD, nonsense mediated decay). The grey lines mark connections between processes. The colored circles represent the number of individual RNA helicases involved in a given process. RNA helicases (yeast and human orthologs) are grouped and color-coded according to their families (see legend at lower left corner). Connectors indicate involvement in one or more processes of RNA metabolism. Clear assignment of SUV3 to either SF is not possible, even though the protein is highly conserved throughout evolution (Fairman-Williams et al., 2010). Circles with bold lines emphasize the three RNA helicases (Prp22p, Prp43p, eIF4A-III) for which specific binding site information is available. Adapted with permission from Elsevier (Jankowsky, 2011).

The Ski2-like helicase Brr2p has been found to unwind base-paired U4/U6 in vitro (Laggerbauer et al., 1998). Unwinding rate and amplitude were significantly reduced in the absence of 3' unpaired regions of U4/U6, suggesting a requirement for overhangs 3' to the
duplex (Pena et al., 2009). Binding of Snu13 to a helical junction in U4 largely protected the U4/U6 structure, and U4 has thus been suggested as the loading strand for unwinding (Pena et al., 2009).

In any of these models with authentic RNA substrates, the role of unwinding by RNA “helicases” has not been unambiguously established and needs more quantitative investigation. Other activities, such as differential RNA binding, strand annealing and strand exchange remain possible.

1.5.3 Protein displacement

The *S. cerevisiae* DEAD-box protein Dbp5p displaced the nuclear poly(A)-binding protein Nab2p from RNA *in vitro* (Tran et al., 2007). Consistently, *in vivo* studies with *nab2* and *dbp5* mutants indicated Nab2p-bound mRNP as the cellular target of Dbp5p (Tran et al., 2007). ADP, but not the non-hydrolyzable ATP analog ADPNP, promoted Dbp5p-catalyzed dissociation of Nab2p (Tran et al., 2007). These observations led the authors to argue that protein displacement by Dbp5p mechanistically differs from *in vitro* ATP-dependent protein-displacement activity shown for the DEAD-box protein Ded1p and the NS3/NPH-II protein NPH-II (Jankowsky et al., 2001; Fairman et al., 2004; Bowers et al., 2005). Since the reactions were performed with enzyme in excess over RNA, it is not known whether ATP is necessary for multiple cycles of Dbp5p-mediated Nab2p removal, and whether there is residual ATP in the ADP preparation that is sufficient for the reaction. Dbp5p has also been implicated in the dissociation of the export receptor Mex67p from mRNPs following export (Lund and Guthrie, 2005). The protein-displacement activity is thought to comprise at least one function of Dbp5p during mRNA export.
The spliceosomal DEAD-box protein Prp28p is required for switching the 5’ splice site from base pairing with U1 snRNA to base pairing with U6 snRNA (Staley and Guthrie, 1999). Interaction between U1 snRNA and the protein U1-C also needs to be destabilized for the switch (Chen et al., 2001), although Prp28p has not been directly shown to remove the protein. Another DEAD-box protein, Sub2p (UAP56 in humans) has been implicated in removing proteins during mRNA 3’ end processing coupled to export (Rougemaille et al., 2008).

1.5.4 RNA chaperone activity

Nuclear-encoded DEAD-box proteins such as the S. cerevisiae Mss116p and the Neurospora crassa CYT-19 promote splicing of mitochondrial group I and group II introns. The nature of this RNA chaperone activity has been under debate. On one hand, it has been suggested that the unwinding activity is used to remodel the introns (Del Campo et al., 2007). On the other, it has been proposed that the ATP-modulated ability to bind RNA is sufficient to stabilize folding intermediates (Solem et al., 2006). In a collaboration with the laboratory of Dr. Alan Lambowitz, we showed that the local strand separation mode of duplex unwinding used by DEAD-box proteins is required for their chaperone activities in group I and group II intron splicing (Del Campo et al., 2009). In contrast, the translocating NS3/NPH-II helicase NPH-II did not promote splicing either in vitro or when introduced into mitochondria. Among DEAD-box proteins, unwinding efficiency correlates with chaperone activity. A recent single-molecule FRET analysis identified multiple steps in the splicing of the group II intron aI5γ promoted by Mss116p (Karunatilaka et al., 2010). Both ATP-independent and ATP-dependent steps were observed (Karunatilaka et al., 2010), consistent with previous biochemical studies (Halls et al., 2007).
1.5.5 RNA clamps and protein binding platform

The DEAD-box protein eIF4AIII functions as an ATP-dependent RNA-clamp in the exon junction complex (EJC) (Bono et al., 2006; Andersen et al., 2006). Other components of the EJC core, Magoh and Y14 bind to the platform provided by eIF4AIII and a small flexible protein Barentsz (Btz), and lock eIF4AIII in its ATP-bound conformation on the RNA. An EJC is deposited at newly formed splice junctions and remains stably bound to the mRNA until the pioneer round of translation (Le Hir et al., 2000; Le Hir et al., 2001; Lejeune et al., 2002; Dostie and Dreyfuss, 2002). A number of other proteins associate with the EJC core (Tange et al., 2004; Gioge and Moore, 2007). For example, Aly/REF interacts with the EJC core to promote binding of mRNA export factor TAP-p15 (Le Hir et al., 2001). If translation has terminated upstream of the last EJC, the EJC core protein Y14 together with RNPS1 acts to recruit Upf proteins and triggers nonsense-mediated decay (NMD) (Singh and Lykke-Andersen, 2003).

1.6 SF2 RNA helicases and their cofactors

RNA helicases are often found in multi-component complexes in the cell. Cofactors may recruit RNA helicases to a specific target, as well as adjusting the biochemical parameters of the helicases for their function. Studies on the impact of cofactors on RNA helicases are key to the understanding of in vivo activities of these proteins.

The DEAD-box protein eIF4AIII is immobilized on the RNA by two other proteins in the EJC, Magoh and Y14 (Ballut et al., 2005; Bono et al., 2006; Andersen et al., 2006). Crystal structures of EJC show Magoh reaching into the ATP site of eIF4AIII (Bono et al.,
ATP hydrolysis does take place within the EJC; rather, product release (ADP + Pi) is inhibited by interaction with Magoh and Y14 (Nielsen et al., 2009).

The DEAD-box protein Dbp5p is stimulated by the nuclear pore protein Gle1p and its coactivator inositol hexakisphosphatase (IP6) (Alcazar-Roman et al., 2006; Weirich et al., 2006). Both the RNA binding and ATPase activity of Dbp5p are facilitated by Gle1p. IP6 binds Gle1p and enhances the stimulation of Dbp5p. Gle1p binds to the 2nd RecA domain of Dbp5p guided by Dbp5p-specific motifs (Dossani et al., 2009). IP6 seems to bind a surface of Gle1p distinct from the Dbp5p binding site, and current data disfavor a molecular glue model where IP6 binds to a pocket formed between Dbp5p and Gle1p (Alcazar-Roman et al., 2010). Different ATP- and RNA-binding states of Dbp5p influence its binding to Gle1p-IP6 (Alcazar-Roman et al., 2010). On the other hand, human DBP5 can be inhibited by the nucleoporin NUP214 in both RNA binding and ATPase activities (von Moeller et al., 2009). Binding of DBP5 to RNA and to NUP214 are mutually exclusive (von Moeller et al., 2009).

In addition, the DEAD-box protein eIF4A is stimulated by eIF4B, eIF4H and eIF4F in unwinding and ATPase activities (Grifo et al., 1984; Rogers et al., 1999; Rogers et al., 2001b; Korneeva et al., 2005; Oberer et al., 2005). The DEAH/RHA protein Prp43p is stimulated by Ntr1p in unwinding and in release of the excised lariat intron (Tanaka et al., 2007). The Ski2-like helicase Brr2p is stimulated by a fragment of Prp8p in unwinding of the U4/U6 stem, but is partially inhibited in ATPase activity (Maeder et al., 2009).

For the DEAD-box protein UAP56 (Sub2p in yeast), Shen et al. did not observe inhibition or stimulation of unwinding and ATPase activities by its cofactor Aly/REF (Shen et al., 2007). Stimulation of ATPase activity, however, has been reported by Taniguchi et al. (Taniguchi and Ohne, 2008).
Collectively, these observations show that cofactors can inhibit, stimulate, or have no effect on RNA binding, unwinding and ATPase activities of a helicase. Although some knowledge has been gained for the impact of cofactors on RNA helicases, more quantitative and mechanistic information on this subject has been lacking. Chapters 3 and 4 of this thesis report systematic characterization of a native complex containing an RNA helicase (TRAMP), and reveals effects of cofactors on the helicase as well as effects of the helicase on the cofactors.

1.7 Studies on TRAMP and Ded1p in this thesis

1.7.1 The Ski2-like protein Mtr4p in the TRAMP complex

The S. cerevisiae Ski2-like helicase Mtr4p is found in a physiological protein complex called TRAMP (Trf4p/Air2p/Mtr4p polyadenylation), which also contains a poly(A) polymerase Trf4p or Trf5p and a zinc-knuckle protein Air1p or Air2p (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). Polyadenylation of a variety of nuclear RNAs by TRAMP stimulates their processing/degradation by the nuclear exosome (Houseley and Tollervey, 2008; Houseley and Tollervey, 2009; Anderson and Wang, 2009; see Chapter 2 for detailed introduction). In vitro, Mtr4p shows RNA-stimulated ATPase and ATP-dependent unwinding activities (Wang et al., 2008; Bernstein et al., 2008). Unwinding by Mtr4p requires single-stranded regions 3’ to a duplex (Wang et al., 2008; Bernstein et al., 2008).

We chose to study the TRAMP complex for a number of reasons. First, the unwinding activity has not been characterized in detail for any Ski2-like helicase. Second,
Mtr4p is in a physiological protein complex, which contains another enzyme, the poly(A) polymerase Trf4p. Third, a lot is known about the cellular targets and pathways for Mtr4p function (see Chapter 2 for a more detailed introduction). Fourth, studies on the TRAMP complex have been mostly conducted with genetic manipulations of yeast and there is a pressing need for quantitative biochemical insights.

In Chapter 3, I quantitatively analyzed the effects of Mtr4p on the polyadenylation activity of Trf4p and established an unprecedented function for an RNA helicase, the modulation of another enzyme in response to features in the RNA substrate. This activity of Mtr4p ensures addition of an oligo(A) tail of ~4 nt, which likely distinguishes TRAMP-polyadenylated RNAs from mRNAs polyadenylated by Pap1p.

Chapter 4 of this thesis characterizes the unwinding activity of the Ski2-like helicase Mtr4p both by itself and within the TRAMP complex and documents the stimulation of Mtr4p by Trf4p and Air2p in the complex. I further investigate the coordination between the poly(A) polymerase Trf4p and the RNA helicase Mtr4p and show that Trf4p can append short oligo(A) tails (~4 nt) to RNA duplexes for unwinding by Mtr4p. The TRAMP complex thus represents a relationship between two enzymes in RNA metabolism that profoundly influences the activities of each other.

1.7.2 The DEAD-box protein Ded1p

The second part of my thesis work focused on the S. cerevisiae DEAD-box protein Ded1p (Chapters 6-9). Previous work on Ded1p from the laboratory of Eckhard Jankowsky has established an unwinding mechanism distinct from canonical translocation-based unwinding, termed local strand separation (Yang and Jankowsky, 2006; Yang et al., 2007a)
(Chapter 6). Basic questions, in particular the oligomeric state of Ded1p in unwinding, and the impact of ATP hydrolysis states on RNA binding, remain to be answered.

Chapter 7 of this thesis shows that the loading and the unwinding units of Ded1p are separable entities. The loading unit binds to single-stranded regions, thereby recruiting the unwinding unit directly onto duplex regions. This extended our understanding of how the presence of single-stranded regions stimulates unwinding of duplex regions by the DEAD-box protein Ded1p.

Chapter 8 of this thesis examines the number of Ded1p molecules bound to RNA substrates of different length in the presence of analogs for different states of ATP hydrolysis (ADPNP, ADP-BeF₃, ADP-AlF₄). On a given length of single strand, the ground state analog ADP-BeF₃ and the transition state analog ADP-AlF₄ lead to binding of more Ded1p molecules than the non-hydrolyzable analog ADPNP, presumably through Ded1p oligomerization. The ability of ssRNA-bound Ded1p to associate with additional Ded1p molecules provides additional support for the above-mentioned “loading unit” in unwinding.
Chapter 2

Introduction

The TRAMP complex, a key player in nuclear RNA metabolism

2.1 Components of the TRAMP complex and their biochemical activities

2.1.1 The TRAMP complex

The TRAMP complex was originally reported to contain the poly(A) polymerase (PAP) Trf4p, the zinc knuckle protein Air2p and the Ski2-like RNA helicase Mtr4p (LaCava et al., 2005). Two independent studies at the same time showed that both Air2p and its paralog Air1p (45% sequence identity) associated with Trf4p in yeast (Wyers et al., 2005; Vanacova et al., 2005). The presence of either Air1p or Air2p is necessary for the polyadenylation activity of Trf4p in vitro (Wyers et al., 2005; Vanacova et al., 2005). A complex formed by a Trf4p paralog Trf5p (65% sequence identity), together with Air1p and Mtr4p was later named TRAMP5 (Houseley and Tollervey, 2006). TRAMP is thus currently thought to consist of a poly(A) polymerase (PAP), either Trf4p or Trf5p, a zinc knuckle
protein, either Air1p or Air2p, and a Ski2-like RNA helicase Mtr4p (Houseley and Tollervey, 2009; Anderson and Wang, 2009).

2.1.2 Difference between TRAMP- and canonical PAP-mediated polyadenylation

Polyadenylation by TRAMP is distinct from mRNA polyadenylation by canonical poly(A) polymerases (PAP) (Anderson and Wang, 2009). Polyadenylation by PAP stabilizes mRNAs and requires specific sequence signals in the RNAs (Keller, 1995; Scorilas, 2002; Wilusz and Spector, 2010). In contrast, polyadenylation by TRAMP designates RNAs for degradation or processing, and TRAMP polyadenylates a large cross-section of diverse RNAs without shared sequence or apparent secondary structure and without common associated proteins (Houseley and Tollervey, 2008; Anderson and Wang, 2008).

Given the opposite goals of the two polyadenylation processes, RNAs polyadenylated by PAP must be distinct from RNAs polyadenylated by TRAMP. Indeed, recent data show a marked difference in the length of the respective poly(A) tails in vivo. While PAP typically appends several dozen to hundreds of adenylates (Keller, 1995), RNAs adenylated by TRAMP contain significantly shorter poly(A) tails (Grzechnik and Kufel, 2008; Keller, 1995; Lebreton et al., 2008) (Table 1.1). Recent data indicate that the distribution of RNAs polyadenylated by TRAMP shows a clear peak at 4-5 added nucleotides (Wlotzka et al., 2011).
Table 2.1 Short poly(A) tail length of TRAMP target RNAs in *S. cerevisiae*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genotype</th>
<th>RNA</th>
<th>Non-templated oligo(A) tail</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>dis3D55IN</td>
<td>5' ETS</td>
<td>G(A)4&lt;br&gt;(A)3&lt;br&gt;(A)2&lt;br&gt;(A)2&lt;br&gt;(A)18&lt;br&gt;(A)8&lt;br&gt;(A)6&lt;br&gt;(A)4&lt;br&gt;(A)3</td>
<td>Lebreton et al&lt;br&gt;Nature 456, 993 (2008)</td>
</tr>
<tr>
<td></td>
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<td>(A)4&lt;br&gt;(A)3&lt;br&gt;(A)2&lt;br&gt;(A)2</td>
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<td></td>
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<td>(A)4(A)2</td>
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Poly(A) tail length was determined by circular RT-PCR followed by cloning and sequencing for pre-rRNA 5' ETS (external transcribed spacer) fragments from dis3D55IN (RRP44 with inactive exonuclease domain) strain (Lebreton et al., 2008) and for a number of snoRNAs from wildtype and rrp6Δ strains (Grzechnik and Kufel, 2008). Non-templated oligo(A) tails in each sequenced clones were listed.

2.1.3 The poly(A) polymerases Trf4p and Trf5p

*TRF4* was initially identified in a screen for synthetic growth defect with *TOP1*, and thus named topoisomerase 1-requiring function 4 (Sadoff *et al.*, 1995). *TRF5* was isolated as a high copy suppressor of the cold sensitive mutant *trf4-1* in the same study and displays sequence homolog to *TRF4* (Sadoff *et al.*, 1995). The *trf4Δ trf5Δ* double mutant is inviable (Sadoff *et al.*, 1995; Wang *et al.*, 2000; Pan *et al.*, 2006). Homologs of Trf4p and Trf5p are found throughout eukaryotes (Stevenson *et al.*, 2006). There are at least 7 potential orthologs of Trf4/5p in humans, and it is currently not clear which of these proteins form a TRAMP-like complex (Stevenson *et al.*, 2006).

Trf4p and Trf5p belong to the Cid1 family of non-canonical poly(A) polymerases (Stevenson *et al.*, 2006). Proteins in the Cid1 family share the NTP transferase domain (also
referred to as the catalytic domain) and the PAP-associated domain (also referred to as the central domain) found in canonical PAPs, but lack an RNA recognition motif (RRM) (Stevenson et al., 2006). Since the RRM in canonical PAPs significantly contributes to binding of the RNA substrate, additional proteins such as Air1/2p, GLD-3 and CPSF are likely involved in RNA binding by the non-canonical PAPs (Stevenson et al., 2006). All PAPs belong to the DNA polymerase β superfamily that also includes other RNA-specific ribonucleotidyl transferases such as CCA-adding enzymes, terminal uridylic transferases and 2’-5’ oligo(A) synthetases (Martin and Keller, 2007).

A recent crystal structure of Trf4p shows an NTP transferase domain (catalytic domain) and a PAP-associated domain (central domain) similar to other members of the DNA polymerase β superfamily (Hamill et al., 2010) (Figure 2.1). The N- and the C-termini are absent from the construct used. The three conserved aspartate residues (D236, D238, D293) lie in the expected positions in the NTP transferase domain (catalytic domain), indicating that the catalytic center is formed between the two domains, with the PAP-associated domain (central domain) making major contributions to ATP binding. Detailed information for the catalytic center of Trf4p may be inferred from structures of canonical PAPs (Martin et al., 2000; Balbo and Bohm, 2007) (Figure 2.2). Several adenine-specific hydrogen bonds were observed in the structure of bovine PAP with 3’ dATP (Figure 2.2A). The yeast PAP^{D154A} in complex with ATP and 5 nt oligo(A), however, displays a different orientation of the adenine base, forming a stacking interaction with the 3’ end of the RNA (Figure 2.2B). Catalytically, Trf4p probably also acts via a two-metal-ion mechanism originally proposed according to crystal structures of DNA polymerases (Steitz, 1998). One magnesium ion coordinates the 3’-hydroxyl group of the RNA substrate attacking on the α-
phosphate of ATP; the other magnesium ion binds all phosphates of ATP and leaves with PP$_i$ (Martin and Keller, 2007).

**Figure 2.1 Crystal structure of the core domains of Trf4p with a fragment of Air2p (Hamill et al., 2010).**

(A) Schematic showing domain organization of Trf4p and Air2p. For Trf4p (161-481 aa), the NTP-transferase (catalytic) domain (161-189, 316-481 aa) is colored light green and the PAP-associated (central) domain (190-315 aa) is colored cyan. Colored boxes in Air2p indicate the five zinc knuckles motifs. A fragment (119-198 aa) containing only the 4th (yellow, 123-136 aa) and the 5th (orange, 164-177 aa) zinc knuckles were used in crystallization.

(B) Ribbon diagram for the partial structure of Trf4p/Air2p. Domains colored as in panel A, with linker regions of Air2p in the same color as their adjacent zinc knuckle motifs. Red spheres indicate zinc atoms. Positions of the three aspartate residues in the catalytic triad are marked in magenta.

Recombinant Trf4p alone does not display poly(A) polymerase activity (Vanacova et al., 2005; Wyers et al., 2005). The presence of either Air1p or Air2p is required for polyadenylation, while conflicting data exist regarding whether Air1p can be added to Trf4p
after purification or needs to be co-expressed with Trf4p (Vanacova et al., 2005; Wyers et al., 2005).

![Figure 2.2 Catalytic center of a PAP.](image)

(A) A bovine canonical PAP with 3'-dATP bound (Martin et al., 2000). The NTP-transferase (catalytic) domain, the PAP-associated (central) domain and the three catalytic aspartate residues are colored as in Figure 2.1. The RRM is removed for clarity. The 3'-dATP is in dark blue, and an additional triphosphate moiety is shown in grey. Residues involved in hydrogen bonding interactions (dashed lines) with the 3'-dATP are shown in stick representation. Red spheres represent Mn$^{2+}$ ions, pink spheres represent water molecules.

(B) *S. cerevisiae* canonical PAP (Pap1p) with ATP and 5 nt oligo(A) bound (Balbo and Bohm, 2007). Asp154 was mutated to Ala to inhibit catalysis. Color scheme as in panel A except that the red sphere indicates a Mg$^{2+}$ ion. Only the 3' nucleotide of the RNA is shown in tan. The path of the RNA extends beneath the region shown.

Trf4p was initially reported to have DNA polymerase and DNA exonuclease activities (Wang et al., 2000; Wang et al., 2002), although these observations were later disputed (Haracska et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). A 5'-deoxyribose-5-phosphate lyase activity was also observed, suggesting a potential role of Trf4p in base excision DNA repair (Gellon et al., 2008).

I am not aware of studies on recombinant Trf5p. TAP-Trf5p (TAP: Tandem Affinity Purification) from *S. cerevisiae* showed less pronounced polyadenylation activity than TAP-Trf4p (Houseley and Tollervey, 2006). *In vivo*, *trf5Δ* is synthetic lethal with *trf4Δ* (Sadoff et
al., 1995; Wang et al., 2000; Pan et al., 2006), and Trf5p and Trf4p have been suggested to play different roles in the cell (Houseley and Tollervey, 2006; Egecioglu et al., 2006; San Paolo et al., 2009). Consistent with distinct functions of Trf4p and Trf5p, a global analysis of protein localization found Trf4p to be throughout the nucleus and Trf5p predominantly in the nucleolus (Huh et al., 2003).

2.1.4 The zinc-knuckle proteins Air1p and Air2p

Air1p and Air2p were named after their interaction with the arginine methyltransferase Hmt1p (Rmt1p) (Arginine methyltransferase-interacting RING-finger protein) (Inoue et al., 2000). Both Air1p and Air2p have five CCHC (C: cysteine, H: histidine) zinc knuckle motifs located between extended N- and C-terminal sequences that are predicted to be unstructured. Air1p and Air2p have been proposed to function in RNA binding (Houseley and Tollervey, 2009; Anderson and Wang, 2009), based on studies of retroviral nucleocapsid proteins that share similar zinc knuckle motifs (D’Souza and Summers, 2005).

A fragment of Air2p containing the fourth and the fifth zinc knuckle motifs were co-crystalized with Trf4p (Hamill et al., 2010) (Figure 2.1). The Air2p fragment interacts with the PAP-associated domain (central domain) of Trf4p. The interaction surface is conserved among Trf4p homologs from yeast to human. In Air2p, the interaction involves the fifth zinc knuckle and a linker that follows (Figure 2.1). The fifth zinc knuckle is thus responsible for protein-protein interaction instead of RNA binding. Residues in the fourth zinc knuckle are available for potential RNA binding (Hamill et al., 2010) (Figure 2.1).

Polyadenylation activity of the Trf4p/Air2p complex is significantly impaired upon deletion of the three N-terminal zinc knuckles from Air2p, with individual deletion of the
first zinc knuckle being the most deleterious among the three (Hamill et al., 2010). The fifth zinc knuckle contacts Trf4p (Hamill et al., 2010) (Figure 2.1), and Air2p with mutations in the fifth zinc knuckle (air2-20) abolished the polyadenylation activity of Trf4p/Air2p (Xuying Wang, Marquette University, unpublished observations).

Air1p inhibits Hmt1p (Rmt1p)-mediated methylation of Npl3p (hnRNP A1 in human) in vitro (Inoue et al., 2000). The protein Npl3p is involved in multiple processes including mRNA export (Lee et al., 1996; Windgassen et al., 2004; Dermody et al., 2008; Kress et al., 2008). While single deletions of AIR1 or AIR2 did not affect cell growth, an air1Δ air2Δ double deletion strain displayed a strong growth defect and accumulated poly(A)$^+$ RNA (hybridized to oligo(dT)$_{50}$) in the nucleus (Inoue et al., 2000). These findings have lead to the proposal that Air1p and Air2p regulate mRNA export through inhibition of Npl3p methylation (Inoue et al., 2000). It has been further suggested that Air1p and Air2p may reduce maturation and export of aberrant mRNPs and direct them to the TRAMP/exosome pathway for degradation (Anderson and Wang, 2009). In addition to Npl3p, Hmt1p (Rmt1p) is involved in methylating hnRNP Hrp1p, U1 snRNP protein Snp1p, and ribosomal protein Rps2p, etc. (Shen et al., 1998; Chen et al., 2010; Lipson et al., 2010). The human homolog of Hmt1p (Rmt1p), PRMT1 (Lin et al., 1996), is responsible for methylating histone H4 at arginine 3 (Wang et al., 2001). A potential effect of Air1p or Air2p on Hmt1p (Rmt1p) activity with these proteins has not yet been tested.

2.1.5 The Ski2-like helicase Mtr4p

The Ski2-like helicase Mtr4p (Dob1p) was identified in a screen for temperature-sensitive mutants that accumulate poly(A)$^+$ RNA in the nucleus (mRNA transport 4) (Kadowaki et al., 1994). Mtr4p is highly conserved from yeast to humans (Jankowsky et al.,
2011). It was the first established cofactor for the nuclear exosome (de la Cruz et al., 1998) (Section 2.3).

As an SF2 helicase, Mtr4p contains a helicase core formed by two RecA-like domains (Jackson et al., 2010; Weir et al., 2010) (Figure 2.3). Additional domains include a WH domain (winged-helix domain), an arch domain (also called the stalk and the KOW domains (Kyprides, Ouzounis, Woese domain)) and a helical bundle domain (Jackson et al., 2010; Weir et al., 2010) (Figure 2.3). The WH domain and the helical bundle domain pack against the RNA binding surface of the helicase core, forming a channel for ssRNA (Jackson et al., 2010; Weir et al., 2010). The surface at the exit of the channel fits the dimensions of the exosome core and is conserved in Mtr4p as well as Ski2p, a cofactor for the cytosolic exosome (Jackson et al., 2010; Weir et al., 2010). Mtr4p has thus been proposed to directly feed the RNA into the central channel of the exosome (Jackson et al., 2010). The prominent arch domain (the stalk and KOW domains) is formed by residues inserted into the WH domain. This large insertion extends beyond the rest of the protein and appears highly flexible. Results from Weir et al. suggest that the stalk domain is not required for interaction with Trf4p/Air2p, and that the KOW domain is required for binding to tRNA\textsubscript{i}^{Met} (Weir et al., 2010). Duplex binding modeled according to a DNA-bound structure of Hel308 (Buttner et al., 2007) is consistent with the KOW domain binding to structured RNA (Jackson et al., 2010; Weir et al., 2010) (Figure 2.3C). The model also supports an unwinding role of the β-hairpin structure from the second RecA-like domain (Section 1.3.3).
Figure 2.3 Crystal structure of Mtr4p with ADP and RNA (Weir et al., 2010).
(A) Domain structure of Mtr4p (Jackson et al., 2010; Weir et al., 2010). Domain names are shown.
(B) Structure of the *S. cerevisiae* Mtr4-Δ80p (residues 81-1073) with ADP and 5 nt poly(A). The helicase core is formed by the two RecA domains (in light blue) together with the N-terminal β-hairpin (in blue), the WH domain (in yellow), and the helical bundle domain (in pink). An insert in the sequence of the WH domain folds into a long stalk (in orange) and a globular KOW domain (in red). ADP and five nucleotides of a single-stranded poly(A) RNA are shown (in ball-and-stick representation in black) bound at the helicase core.
(C) Model of Mtr4-Δ80p binding to double-stranded nucleic acid. The structure shown in panel B was modeled with the partially unwound nucleic acid in the Hel308 structure (Buttner et al., 2007), after superposition of the two helicase core regions. The single-stranded portion of the 3′ product strand superposes well with the equivalent five ribonucleotides of single-stranded RNA present in the Mtr4p structure. The duplex region is near the equivalent unwinding β-hairpin of Mtr4p and near the KOW domain.
Modified with permission from the authors (Weir et al., 2010).
The structure of Mtr4p from Weir et al. was bound to 5 nt oligo(A) (Weir et al., 2010). However, no base-specific hydrogen bonds were observed except with the incomplete molecule B. Interestingly, in molecule B, E947 from the helical bundle domain makes a single hydrogen bond with N6 of the fourth adenine from the 5’ end, which constitutes the only adenine-specific recognition (Figure 2.4). R1026 and R1030 from the helical bundle domain make purine-specific contacts to the fourth and the fifth adenines (Figure 2.4). These interactions suggest a particular significance for the fourth and the fifth adenines in Mtr4p (TRAMP) function (Chapters 3-5).

Figure 2.4 Recognition of adenine sequences by Mtr4p. Only residues 945-951 and 1026-1036 in the helical bundle domain of Molecule B from Weir et al. (Weir et al., 2010) are shown (in gray). E947 establishes a single H-bond with N6 of the 4th adenine from the 5’ end (adenine specific). R1026 H-bonds to N7 in the 4th and the 5th adenine (purine specific) and to the 4th phosphate. R1030 forms a single H-bond to N3 in the 5th adenine (purine specific).

Mtr4p contains a Q-motif (Section 1.3.1), and thus either ATP or dATP is required for NTP hydrolysis and RNA duplex unwinding (Bernstein et al., 2008; Wang et al., 2008). A single-stranded region 3’ to the duplex is required for unwinding by Mtr4p (Bernstein et al., 2008; Wang et al., 2008), as with most non-DEAD-box SF2 helicases (Section 1.4).
Mtr4p binds oligo(A) sequences (5, 10, 20 nt) at least 2-fold tighter than random sequences of the same length (Bernstein et al., 2008; Bernstein et al., 2010). The affinity of Mtr4p for RNA was also modulated by the presence of ADP or ADPNP (Bernstein et al., 2008; Bernstein et al., 2010).

2.2 The 3’->5’ exonuclease complex exosome

Degradation/processing of TRAMP target RNAs is achieved by a 3’->5’ exonuclease complex, the nuclear exosome (Houseley and Tollervey, 2008; Anderson and Wang, 2009). In S. cerevisiae, the exosome contains a hexameric ring of RNase PH domain proteins, Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p (Houseley et al., 2006). Similar structures are found in all three domains of life (bacteria, archaea, eukaryota). However, PH domain proteins in yeast have apparently lost RNase activity and only the Rrp41/Rrp45 subcomplex exhibited activity in humans (Houseley et al., 2006; Liu et al., 2006). According to structures of human and archaeal exosomes, three S1/KH domain proteins Rrp4p, Rrp40p and Csl4p form an RNA entry pore on one side of the hexameric ring (Liu et al., 2006; Buttner et al., 2005). A homolog of E. coli RNase R and RNase II, Rrp44p (Dis3p), binds on the opposite side of the hexameric ring, contacting Rrp41p and Rrp45p (Bonneau et al., 2009). Besides the RNase II-like domain, a PIN (PiIT N-terminal) domain has been identified in the N-terminus of Rrp44p, and catalyzes endonucleolytic cleavage of single-stranded RNA (Lebreton et al., 2008; Schaeffer et al., 2009; Schneider et al., 2009). The above-mentioned 10 proteins are found in both the nuclear and the cytoplasmic forms of the exosome. The nuclear exosome also contains a non-essential RNase D homolog Rrp6p (Allmang et al., 1999).
The exonuclease activity of Rrp44p is inhibited in the presence of the 9-component exosome core, through contacts with Rrp45p (Liu et al., 2006; Bonneau et al., 2009). Activating cofactors for the nuclear exosome include Mtr4p, the TRAMP complex, Rrp47p, and Nrd1p together with Nab3p, Sen1p and the cap binding complex (Sto1p and Cbc2p) (Houseley et al., 2006). The mechanistic nature of cofactor functions is largely unclear, but it almost certainly includes recognition of the various RNA substrates (Section 2.5). For TRAMP (TAP-purified from S. cerevisiae), a recent study reported that TAP-TRAMP does not stimulate RNA degradation by the core exosome containing Rrp44p, but enhances the activity of Rrp6p (Callahan and Butler, 2010). This stimulation of Rrp6p activity by TRAMP does not depend on Mtr4p (Callahan and Butler, 2010).

The cytoplasmic exosome relies on the Ski complex (Ski2p, Ski3p, Ski8p) for all mRNA degradation pathways (Brown et al., 2008; Houseley et al., 2006). An eRF3-like domain-containing protein Ski7p associates with the cytoplasmic exosome and is thought to bind to stalled ribosomes and target mRNAs for degradation (van Hoof et al., 2002). Nonsense-mediated decay requires another complex formed by Upf1p, Nmd2p (Upf2p) and Upf3p (He et al., 1997).

2.3 Difference between the TRAMP/exosome pathway and the bacterial degradosome

Until the discovery of TRAMP in 2005 (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005), poly(A) tails in eukaryotes were long thought to be a stabilizing signal restricted to mRNAs. In bacteria, however, a role of polyadenylation in degradation was described a decade earlier (Ehretsmann et al., 1992; Carpousis et al., 1994; Causton et al., 1994, Py et al., 1994). In E. coli, the poly(A) polymerase (PAP) facilitates mRNA
degradation by a protein complex termed the degradosome (Carpousis, 2007). The degradosome contains one essential component, RNase E, and multiple major components such as PNPase (polynucleotidyl phosphorylase), RhlB (RNA helicase B) and enolase (Miczak et al., 1996; Py et al., 1996).

While functionally analogous, the E. coli degradosome differs from the S. cerevisiae TRAMP and exosome in a number of ways. PAP loosely associates with the endonuclease RNase E, the significance of which is unclear (Carpousis, 2007). No stable interaction between PAP and the helicase RhlB has been observed (Carpousis, 2007). RhlB is a DEAD-box protein instead of a Ski2-like helicase. Other DEAD-box proteins, CsdA, RhlE and SrmB can also bind RNase E, at a site distinct from the binding site for RhlB (Khemici et al., 2004). RhlB shows ATPase and RNA duplex unwinding activities only in the presence of a peptide from RNase E, which simulates the ATPase activity and participates in RNA binding (Vanzo et al., 1998; Khemici et al., 2004; Chandran et al., 2007). Exonucleases PNPase, RNase II and RNase R all function after endonuclease cleavage of polyribosomal mRNA by RNase E. The eubacterial PNPase is a homotrimer with six PH domains and three active sites, while the yeast exosome core has six PH domains in six proteins that are all inactive (Houseley et al., 2006). Moreover, PNPase catalyzes the reverse reaction and functions as a poly(A) polymerase (Mohanty and Kushner, 2000). The function of enolase in the degradosome is unclear, but it is likely that enolase provides a link between the energetic states of the cell and mRNA degradation (Carpousis, 2007).

Despite these differences, polyadenylation activates degradation of structured RNAs by the degradosome (Xu and Cohen, 1995; Coburn and Mackie, 1996), and the oligo(A) tails are no more than 5 nt in vitro and in vivo (Xu et al., 1993; Blum et al., 1999). These
similarities to the TRAMP/exosome pathway suggest common constraints posed by RNA degradation. The mechanism by which polyadenylation and degradation are linked, however, probably differs in quite fundamental ways between bacteria and eukaryotes.

2.4 RNA substrates of the TRAMP complex

The TRAMP complex has been implicated in processing or degradation of an astounding variety of RNAs in the nucleus. This section summarizes current knowledge of TRAMP targets, based largely on genetic studies.

2.4.1 Ribosomal RNAs (rRNAs)

Among the first phenotypes for MTR4 mutants were defects in 3’ processing of 5.8S rRNA from its 7S precursor (de la Cruz et al., 1998) and accumulation of poly(A)+ RNAs close to the nucleolus (Liang et al., 1996). Another well-characterized rRNA target of Mtr4p is the 5’ ETS (external transcribed spacer) of the tricistronic pre-rRNA (precursor of 18S, 5.8S and 28S) (de la Cruz et al., 1998). Upon deletion of the nuclear-specific exosome component RRP6, polyadenylated forms were detected for pre-rRNAs 35S, 32S, 27S, 23S, 21S, 20S, 17S’, 7S and 5.8S+30, and for mature 25S and 18S rRNAs (Houseley and Tollervey, 2006). These species showed significant reductions in polyadenylation in an rrp6Δ trf4Δ strain and an rrp6Δ trf5Δ strain (a trf4Δ trf5Δ strain is lethal, as discussed in Section 2.1.3) (LaCava et al., 2005; Houseley and Tollervey, 2006), suggesting that the RNAs are all polyadenylated by TRAMP. Curiously, depletion of Mtr4p led to hyperadenylation of (pre-)(rRNA, to an extent greater than that observed with depletion of exosome components (Houseley and Tollervey, 2006).
2.4.2 Small nucleolar RNAs (snoRNAs)

A second major class of TRAMP substrates is comprised of snoRNAs. Both box H/ACA class snR46 and box C/D class snR13, snR65, U14 and U24 depend on Trf4p and Trf5p for maturation (LaCava et al., 2006; Houseley and Tollervey, 2006; Grzechnik and Kufel, 2008). Defects in formation of these snoRNAs were also observed in a GAL::MTR4 yeast strain grown in glucose (Mtr4p depletion) and in an air1Δ air2Δ strain (LaCava et al., 2006; Houseley and Tollervey, 2006; Grzechnik and Kufel, 2008). Processing of other snoRNAs of both classes have been shown to depend on Mtr4p and the exosome component Rrp6p and are thus potential substrates of TRAMP as well (van Hoof et al., 2000). Besides a role in polyadenylation, the presence of Trf4p (trf4-236, with mutations D236A,D238A) is required for Nrd1/Nab3-dependent transcription termination of snoRNAs, and Trf4p (TRAMP) appears to enhance Nrd1p association with the transcripts (Grzechnik and Kufel, 2008). The extensive involvement of TRAMP in snoRNA maturation conceivably contributes to phenotypes in rRNA processing.

2.4.3 Transfer RNAs (tRNAs)

A hypomethylated precursor of tRNA initiator methionine (pre-tRNAi\textsuperscript{Met}) was the first substrate of the TRAMP complex to be characterized (Kadaba et al., 2004; Kadaba et al., 2006). m\textsuperscript{1}A58 methylation in pre-tRNAi\textsuperscript{Met} is likely crucial for tertiary folding and yeast containing a mutation in the m\textsuperscript{1}A methyltransferase gene TRM6 (trm6-504) shows a reduced level of mature tRNAi\textsuperscript{Met} at non-permissive temperatures (Anderson et al., 1998; Kadaba et al., 2004). This phenotype was suppressed by mutations in either the exosome component Rrp44p (rrp44-20), or the TRAMP components Trf4p (trf4-20) or Mtr4p (mtr4-20) (Kadaba
et al., 2004; Wang et al., 2008). Current evidence suggests that tRNAs become substrates of TRAMP-mediated surveillance not only when hypomodified, but also when their 3’ end processing or intron removal is perturbed (Anderson and Wang, 2009; Copela et al., 2008).

2.4.4 Small nuclear RNAs (snRNAs)

RNA targets of TRAMP also include U4 snRNA (Egecioglu et al., 2006) and U5 snRNA (LaCava et al., 2005), wildtype U6 (Wyers et al., 2005) and truncated U6 snRNA (snr6Δ59-72) (Kadaba et al., 2006).

2.4.5 Long transcripts of RNA polymerase II (Pol II)

An mRNA-like potential substrate of TRAMP was detected in the first study of Mtr4p (Kadowaki et al., 1994). CRY1 mRNA displayed an extended form in the presence of a mutation in Mtr4p (mtr4-1), as well as mutations in exosome components Mtr3p (mtr3-1) and Rrp44 (mtr17-1) (Kadowaki et al., 1994). While this extended mRNA was not characterized in yeast, results from mammalian cells hint at a possible involvement of TRAMP in transcription termination of certain mRNAs. Human β-globin pre-mRNA is cotranscriptionally cleaved (CoTC) preceding transcription termination (Dye and Proudfoot, 2001). The 5’ product of the cleavage is stabilized by depletion of exosome components (PM/Scl-100, PM/Scl-75, homologs of Rrp6p, Rrp45p respectively) and contains short oligo(A) tails, raising the possibility that the 5’ cleavage product is a substrate of a human TRAMP-like complex (West et al., 2006). Moreover, cryptic unstable transcripts (CUTs) transcribed from intergenic regions as well as promoter-associated transcripts are polyadenylated by Trf4p (TRAMP) and degraded by the nuclear exosome (Wyers et al., 2005; Davis and Ares, Jr., 2005). A recent study of RNAs that cross-linked to Trf4p
identified thousands of mRNAs along with antisense and intergenic transcripts (Wlotzka et al., 2011). Trf4p and Trf5p also promote degradation of spliced-out introns (San Paolo et al., 2009). Overexpression of a mutant Trf4p (Trf4-236p) diminished the accumulation of most introns seen in a \textit{trf4Δ} strain, suggesting that Trf4p facilitates degradation of introns independent of polyadenylation (San Paolo et al., 2009).

\textbf{2.4.6 Mechanism of substrate identification by TRAMP is unknown}

Collectively, these reports show that the TRAMP complex is involved in processing/surveillance of a wide range of RNAs produced by all the three RNA polymerases (Pol I, II, III). The broad spectrum of RNA substrates poses the question how TRAMP identifies and acts on its targets. The canonical PAP relies on another protein, CPSF, to recognize the AAUAAA signal for mRNA polyadenylation. No such factor or signal is known for TRAMP, although Nrd1/Nab3 have recently been proposed to function upstream of TRAMP on both Pol II and Pol III transcripts (Wlotzka et al., 2011). Substrate recognition by the TRAMP/exosome pathway remains a central question for understanding TRAMP function.

\textbf{2.5 TRAMP and chromatin maintenance}

Since the initial identification of \textit{TRF4 (trf4-1)} in a genetic screen with a DNA topoisomerase I deletion (\textit{top1Δ}) (Sadoff et al., 1995), the list of genetic and physical interactions between TRAMP components and proteins in DNA metabolism has significantly expanded (Table 2.2). While the direct action of TRAMP on DNA substrates remains possible (Section 2.1), current evidence points to a role in chromatin maintenance through
RNA. TRAMP target RNAs are transcribed from across the genome, and the act or the products of transcription likely impact chromatin structures. An indirect effect through postranscriptional modifications mediated by enzymes such as the methyltransferase Hmt1p (Rmt1p) is also possible (Section 2.1.4). A few studied examples of the role(s) of TRAMP in chromatin maintenance are summarized below.

In a trf4Δ yeast strain, cryptic transcripts were detected from repressed chromatin regions including a telomeric region (TEL05L), the rDNA intergenic spacer region (IGS), a centromeric region (CEN3) and the silenced mating-type cassettes (MATa/α) (Houseley et al., 2007). The transcript from the IGS (IGS1-R) passes through the replication fork barrier, a region required for rDNA copy number control, explaining the loss of rDNA repeats in the absence of Trf4p (Houseley et al., 2007) and supporting early observations of rDNA hyperrecombination in the trf4-1 mutant (Castano et al., 1996, Sadoff et al., 1995). A trf4Δ strain showed a more severe telomere shortening defect than that observed in a rrp6Δ strain (nuclear exosome component), and is partially rescued by enzymatically inactive TRF4 (trf4-236) (San Paolo et al., 2009). The authors attributed the phenomenon to an imbalance between telomeric proteins and telomeric RNA (TLC1).

In Schizosaccharomyces pombe, the Trf4p and Trf5p homolog Cid14 is required for silencing of centromeric, sub-telomeric regions and for maintenance of rDNA (Wang et al., 2008). With Cid14 deleted, TRAMP substrates spuriously associate with Ago1, a component of the RNA-induced transcriptional silencing (RITS) and the Argonaute siRNA chaperone (ARC) complexes, thus interfering with siRNA-mediated silencing of heterochromatic regions (Buhler et al., 2008). The protein Mlo3 (Yra1p in S. cerevisiae, Aly/REF in
mammals) associated with both TRAMP and the Clr4/RITS complex, and a cooperation between the TRAMP/exosome pathway and RNAi has been proposed (Zhang et al., 2011).

Given the effects of TRAMP on chromatin silencing, it is worth noting that accumulation of an RNA species upon deletion or mutation of a TRAMP or exosome component does not establish a *bona fide* direct target of the TRAMP/exosome pathway without mechanistic analyses. Future studies on the role(s) of TRAMP in DNA metabolism might reveal extensive involvement of noncoding RNAs in shaping the eukaryotic genome.
Table 2.2 Genetic and physical interactions between TRAMP components and proteins in DNA metabolism.

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Data source *Saccharomyces* Genome Database (SGD). The SGD alias TRF4 is used in place of PAP2 for consistency with the text. For meanings of gene ontology (GO) terms and genes involved, see [http://www.yeastgenome.org/cgi-bin/GO/goTerm.pl](http://www.yeastgenome.org/cgi-bin/GO/goTerm.pl) and [http://amigo.geneontology.org/cgi-bin/amigo/go.cgi](http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). Related processes with extensive involvement of TRAMP components are shaded. Interactions with proteins involved in the metabolism of the DNA base are not included.
2.7 Biochemical characterization of the TRAMP complex

How polyadenylation by TRAMP is controlled is unknown and not apparent from the current model of TRAMP function. In this model, Trf4p/5p acts as the principal poly(A) polymerase, assisted by Air1/2p, which is required for Trf4p activity in vitro (Anderson and Wang, 2009; Houseley and Tollervey, 2008). The Ski2-like RNA helicase Mtr4p is thought to subsequently unwind RNA duplexes with 3’ to 5’ polarity (Wang et al., 2008). Mtr4p (Dob1p) was the first factor implicated in aiding RNA degradation by the exosome (de la Cruz et al., 1998). It is thought that the unwinding activity of Mtr4p, shown for the recombinant protein, stimulates RNA degradation (Bernstein et al., 2008; Wang et al., 2008; Anderson and Wang, 2009; Houseley and Tollervey, 2008). This basic model of TRAMP function has been instructive in assigning enzymatic roles to the components. However, the model cannot explain more intricate TRAMP functions, such as the control of poly(A) tail lengths. Most likely, the yet unexplored interplay between the TRAMP components gives rise to these functions.

In Chapters 3 and 4, I investigate the cross-talk between the poly(A) polymerase Trf4p and the RNA helicase Mtr4p by quantitatively analyzing polyadenylation and unwinding reactions with TRAMP. This study reveals effects of Mtr4p on the polyadenylation activity of Trf4p and effects of Trf4p/Air2p on the helicase activity of Mtr4p.
Chapter 3

The RNA helicase Mtr4p modulates polyadenylation in the TRAMP complex

3.1 Introduction

To examine the interplay between the TRAMP components and to illuminate the molecular basis of the poly(A) length control, I quantitatively analyzed individual adenylation steps by wtTRAMP (Trf4p/Air2p/Mtr4p), Trf4p/Air2p and TRAMP with mutant Mtr4p (TRAMP$^{Mtr4p-20}$, TRAMP$^{Mtr4p(E947A)}$). Various aspects of the polyadenylation reaction, including adenylation rate constants, affinities for RNA substrates and ATP, processivity and dissociation rate constants were examined for each adenylation step.

3.2 Results

3.2.1 Reconstitution and purification of the S. cerevisiae TRAMP complex from proteins expressed in E. coli

The recombinant TRAMP complex was reconstituted and purified from an E. coli strain co-expressing Trf4p/Air2p and an E. coli strain expressing Mtr4p. Lysates from the two strains were combined in volumes containing a roughly five-fold molar excess of Mtr4p over Trf4p/Air2p to ensure complete formation of the TRAMP complex. Affinity purification against His$_6$-tagged Mtr4p and Air2p was followed by affinity purification against FLAG-tagged Trf4p. The reconstituted TRAMP complex contained all the three components in
stoichiometric amounts and the integrity of the complex was verified by gel filtration and sucrose gradient sedimentation (Figure 3.1 and data not shown).

3.2.2 TRAMP displays modulated polyadenylation activity

Polyadenylation activity of the reconstituted TRAMP was first measured using \textit{in vitro} transcribed tRNA$_{\text{Met}}$ (Figure 3.2A). This RNA resembles one of the physiological targets of TRAMP, hypomethylated pre-tRNA$_{\text{Met}}$ (Section 2.4.3), and had been previously used to detect polyadenylation activity of TRAMP obtained from yeast (Kadaba \textit{et al.}, 2004; Wang \textit{et al.}, 2008). Reactions were performed under pre-steady state conditions with enzyme in excess over the substrate. The kinetic description of pre-steady reactions contains fewer parameters than steady-state regimes and thus provides the most accurate quantitative data.
Polyadenylation time courses were analyzed by denaturing PAGE to resolve polyadenylated species at single nucleotide resolution (Figure 3.2A).

![Diagram](image)

**Figure 3.2** Polyadenylation of tRNA\textsubscript{Met} by TRAMP displays temporary accumulation of species with 3-5 A.

(A) Polyadenylation reaction with radiolabeled (asterisk) tRNA\textsubscript{Met} (0.5 nM tRNA\textsubscript{Met}, 150 nM TRAMP, 2 mM equimolar ATP-Mg\textsuperscript{2+}). Aliquots were removed at 1 min intervals and resolved by denaturing PAGE. Added adenylates are marked on the right.

(B) Left: contour plot of the fraction of the adenylated intermediates (A\textsubscript{i}) vs. reaction time for the time course in panel A. The color bar shows the color progression from A\textsubscript{i} = 0 to 0.2 (contours: A\textsubscript{i} = 0.035, 0.070, 0.105, 0.140, 0.175). Right: contour plot for a simulated reaction with equal rate constants for each adenylation step (k = 1.5 min\textsuperscript{-1}).

Plots of the fractions of polyadenylated species vs. reaction time revealed accumulation of species with 3 to 5 adenylates in a time window from approximately 1 to 3 min (Figure 3.2B). Over longer reaction times, the poly(A) tail grew to 15 nt and longer (Figure 3.2). The temporary accumulation of species with 3-5 adenylates suggested a modulation of the polyadenylation activity in response to the number of added nucleotides.

To test this assertion, we determined rate constants for individual adenylation steps. The polyadenylation reaction was described as a series of irreversible, pseudo-first-order addition of adenylate residues. The observed rate constants for individual adenylation steps
\(k_{\text{obs}}\) represent multiple physical processes, including adenylation and dissociation of TRAMP from the RNA. An analytical mathematical model for the reaction scheme was implemented into a computer routine that performs non-linear regression with experimentally measured time courses to determine individual adenylation rate constants (see Appendix 3 for details). The rate constants obtained faithfully described the experimental data (Figure 3.3A, B).

![Figure 3.3](image)

**Figure 3.3** Quantitative analysis of individual adenylation steps reveals poly(A) length-dependent modulation.
(A) Kinetic scheme for the polyadenylation reaction. For corresponding equations and fitting of the dataset in panel B see Appendix 2.
(B) Plots show representative time courses for selected species (\(A_0, A_1, A_2, A_{11}\)) from the reaction displayed in Figure 3.2A. Lines indicate non-linear regression according to the scheme shown in panel A (see Appendix 2 for details).
(C) Observed rate constants for individual adenylation steps. Points represent averages for multiple independent experiments as shown in Figure 3.2A. The error bars mark one standard deviation.

Plots of rate constants vs. the corresponding number of added adenylates revealed poly(A) length-dependent changes in the adenylation activity. Rate constants increased for the first three steps, and then decreased to a fairly constant level (Figure 3.3C). The resulting peak in rate constants explains the temporary accumulation of RNA species with 3-5
adenylates in a straightforward manner: TRAMP forms these species relatively fast, but extends them only slowly.

Figure 3.4 Modulation of polyadenylation activity is seen regardless of the order of addition of reaction components. Polyadenylation reactions were performed with 0.5 nM tRNA$_{\text{Met}}$, 100 nM wtTRAMP and 2 mM ATP-Mg$_2^+$. Reaction schemes in the upper panels correspond to the plot of individual adenylation rate constants in the lower panels. (A) TRAMP and RNA were incubated in reaction buffer for 5 min, ATP was added to start the reaction. The dashed line marks the peak adenylation rate constant at A$_3$. (B) TRAMP and ATP were incubated in reaction buffer for 5 min, RNA was added to start the reaction. (C) RNA and ATP were incubated together in reaction buffer for 5 min, TRAMP was added to start the reaction.

The increase and subsequent decrease in polyadenylation activity as the poly(A) tail grew was observed regardless of the order in which TRAMP, RNA and ATP was added (Figure 3.4) and over a wide range of TRAMP concentrations (Figure 3.5). To obtain maximum rate constants at TRAMP saturation ($k_{\text{max}}^{\text{TRAMP}}$) and apparent substrate affinities ($K_{1/2, \text{TRAMP}}$), rate constants for individual adenylation steps were plotted vs. TRAMP concentration (Figure 3.5C, D). The peak for adenylation rate constants remained at A$_3$. 
indicating that the polyadenylation activity of TRAMP is modulated even at saturating TRAMP concentrations (Figure 3.5C). Apparent substrate affinities were weaker for early steps and reached low nanomolar range after about 4 steps (Figure 3.5D).

![Figure 3.5](image-url)

**Figure 3.5** Determination of maximal adenylation rate constants and apparent substrate affinities from TRAMP titrations.
(A) Representative plots of observed adenylation rate constants for individual steps at different TRAMP concentrations (open circles: 25 nM TRAMP; filled circles: 250 nM TRAMP). Polyadenylation reactions were performed with 0.5 nM tRNA^{Met}, 2 mM ATP-Mg^2+ and indicated concentrations of TRAMP.
(B) Representative plots of adenylation rate constants for each step vs. TRAMP concentration. Examples shown are for step 1 (filled circles) and step 3 (open circles). For each step, adenylation rate constants were fit to a binding isotherm according to \( k_{\text{obs}} = \frac{k_{\text{max}} \text{TRAMP} [\text{TRAMP}]}{K_{1/2} \text{TRAMP} + [\text{TRAMP}]} \). Rate constants at TRAMP saturation at each step (\( k_{\text{max}} \text{TRAMP} \), panel C) and the TRAMP affinity (\( K_{1/2} \text{TRAMP} \), panel D) were obtained from the fits.
(C) Resulting plot of maximum rate constant at TRAMP saturation. Error bars indicate the standard deviation of the values obtained by fitting the curves in panel B.
(D) Apparent substrate affinity (\( K_{1/2} \text{TRAMP} \)) for individual steps. Error bars indicate the standard deviation of the values obtained by fitting the curves illustrated in panel B.

To gain further insight into the molecular basis of the modulated polyadenylation activity, we examined the dependence of individual rate constants on the ATP concentration.
We determined the functional affinity for ATP ($K_{1/2, ATP}$) for each adenylation step (Figure 3.6) and each adenylation rate constant at ATP saturation ($k_{\text{max}}^{\text{ATP}}$, data not shown). This allowed us to calculate maximum rate constants at both TRAMP and ATP saturation ($k_{\text{max}}$, Figure 3.7). The observed peak in polyadenylation rate constants at A$_3$ broadened slightly at ATP saturation (cf. Figures 3.3C, 3.5C, 3.7B). A pronounced drop in ATP affinity was detected at A$_5$/A$_6$, immediately after the peak for the highest adenylation rate constant at A$_3$ (Figure 3.6B). The decrease in adenylation rate constants is thus accompanied by a marked reduction in ATP affinity. Modulations of both ATP affinity and adenylation rate constants synergistically favor the temporal accumulation of species with 3-5 adenylates.

**Figure 3.6 Determination of maximal adenylation rate constants and apparent ATP affinities from ATP titrations.**
(A) Representative plots of adenylation rate constants for each step vs. ATP concentration (0.5 nM tRNA$_{\text{Met}}$, 100 nM TRAMP). Examples shown are for step 1 (filled circles) and step 3 (open circles). For each step, adenylation rate constants were fit to a binding isotherm according to $k_{\text{obs}} = k_{\text{max}}^{\text{ATP}} [\text{ATP}] / (K_{1/2, \text{ATP}}^{\text{ATP}} + [\text{ATP}])$. Rate constants at ATP saturation ($k_{\text{max}}^{\text{ATP}}$, data not shown) and the ATP affinity ($K_{1/2, \text{ATP}}^{\text{ATP}}$, panel B) at each step were obtained from the fits.
(B) Apparent ATP affinity ($K_{1/2, \text{ATP}}^{\text{ATP}}$) for individual adenylation steps. Error bars indicate the standard deviation of the values obtained by fitting the curves illustrated in panel A.
Figure 3.7 Determination of maximum rate constants at both TRAMP and ATP saturation. (A) Maximum rate constants determined from TRAMP and ATP titrations ($k_{\text{max,TRAMP}}$ and $k_{\text{max,ATP}}$ respectively) extrapolated to TRAMP saturation ($k_{\text{max,TRAMP}}$, open circles) and to ATP saturation ($k_{\text{max,ATP}}$, filled circles). According to the binding isotherms used in Figures 3.5, 3.6, $k_{\text{max,TRAMP}} = k_{\text{max,ATP}} (1 + K_{1/2,\text{TRAMP}}/[\text{ATP}])$; $k_{\text{max,ATP}} = k_{\text{max,TRAMP}} (1 + K_{1/2,\text{ATP}}/[\text{TRAMP}])$. $k_{\text{max,TRAMP}}$ and $K_{1/2,\text{TRAMP}}$ were obtained from TRAMP titrations as illustrated in Figure 3.5; [ATP] is the ATP concentration used in TRAMP titrations. $k_{\text{max,ATP}}$ and $K_{1/2,\text{ATP}}$ were obtained from ATP titrations as illustrated in Figure 3.6, in ways similar to Figure 3.5; [TRAMP] is the TRAMP concentration used in ATP titrations. (B) Rate constants at TRAMP and ATP saturation ($k_{\text{max}}$) for individual adenylation steps. Data represent the average of values determined from different order of extrapolation shown in panel A. The error bars mark one standard deviation.

3.2.3 Hypomethylated pre-tRNA$_i^{\text{Met}}$, a prototypical TRAMP target, accumulates poly(A) tails with approximately 4 adenylates in vivo

We next examined whether the physiological TRAMP target, hypomethylated tRNA$_i^{\text{Met}}$ precursor (pre-tRNA$_i^{\text{Met}}$) (Kadaba et al., 2004; Kadaba et al., 2006), accumulated similarly short poly(A) tails in vivo. To measure the poly(A) tail lengths of cellular pre-tRNA$_i^{\text{Met}}$ with single-base resolution, we adopted a 3’ RACE (Rapid Amplification of cDNA Ends) strategy (Figure 3.8A). We isolated total RNA from the yeast trm6-504 strain, where the non-functional tRNA m$^1$A methyltransferase Trm6p leads to accumulation of hypomethylated pre-tRNA$_i^{\text{Met}}$ which is targeted by TRAMP (Kadaba et al., 2004). Following the extension of the RNA 3’ ends with guanosine-inosine tails, polyadenylated pre-tRNA$_i^{\text{Met}}$ were specifically amplified by RT-PCR. We accounted for the heterogeneity in the 3’ ends of pre-tRNA$_i^{\text{Met}}$ (Kadaba et al., 2004) by processing of the PCR products with the restriction
enzyme MseI, which was possible because all precursors end with at least two 3’ uridines (Figure 3.8A). The isolated pre-tRNA\textsubscript{iMet} poly(A) tails were ligated to a piece of synthetic DNA, amplified and subjected to Sanger sequencing to delineate the number of added adenylates (Figure 3.8A).

The method was calibrated with a tRNA\textsubscript{iMet} processed \textit{in vitro} (gel panel in Figure 3.8B). The corresponding sequencing chromatogram shows excellent agreement between input and final sequencing result (Figure 3.8B). The robustness of the method was further tested with longer, \textit{in vitro} generated poly(A) tail lengths, and similar agreements were seen (data not shown).

We then measured the lengths of poly(A) tails of pre-tRNA\textsubscript{iMet} appended \textit{in vivo} (Figure 3.8C). The corresponding Sanger chromatogram indicates accumulation of RNA species with roughly 4 adenylates (Figure 3.8C), in excellent agreement with our polyadenylation measurements \textit{in vitro} (Figure 3.2). Accumulation of similarly short poly(A) tails on other TRAMP targets \textit{in vivo} had been observed by others (Grzechnik and Kufel, 2008; Lebreton \textit{et al.}, 2008; Wlotzka \textit{et al.}, 2011). The striking correlation between the poly(A) tail lengths distribution of TRAMP targets \textit{in vivo} and the temporary accumulation of short poly(A) tails in \textit{in vitro} is consistent with the notion that TRAMP displays modulated polyadenylation activity in the cell as well.
Figure 3.8 Accumulation of poly(A) tails of approximately 4 adenylates on hypomethylated pre-tRNA$^{\text{Met}}$ in vivo.

(A) Experimental scheme to measure poly(A) tail lengths of pre-tRNA$^{\text{Met}}$ in vivo by Sanger sequencing. Pre-tRNA$^{\text{Met}}$ from genes IMT1-4 contains different 3'-terminal sequences. In vitro transcribed tRNA$^{\text{Met}}$ with ~3 uridines appended was used as control. Pre-tRNA$^{\text{Met}}$ polyadenylated by
TRAMP in vivo (total RNA from trm6-504 strain) or in vitro were incubated with poly(U) polymerase to extend the 3’ ends with a mixture of G:1 ribonucleotides. Next, a tailed RT primer consisting of a stretch of ten cytosines and two terminal thymidines (to anchor the oligonucleotide to the 3’ end of the poly(A) tail) was used for first strand cDNA synthesis. Pre-tRNAMet was subsequently amplified via RT-PCR. To eliminate the heterogeneity in the 3’-terminal sequences of IMT1-4, the pre-tRNAMet moiety of the RT-PCR products was digested with MseI which cuts at the 5’ end of the poly(A) tail of all pre-tRNAiMet. Poly(A) tails were subsequently ligated to a DNA linker and ligation products were specifically amplified by PCR. Sanger sequencing of PCR products was performed with internal sequencing primers. As illustrated here, the expected chromatograms show a gradual decrease in signal for adenine, concomitant with a gradual increase in signal for guanine.

(B) Left panel: in vitro transcribed tRNAiMet with three 3’-terminal uridines polyadenylated by TRAMP (0.5 nM 32P-labeled RNA, 150 nM TRAMP, 2 mM ATP-Mg2+ for 3 min). The number of appended adenylates is marked. Right panel: Representative Sanger sequencing chromatogram for this RNA after the poly(A)-tail length measurement procedure shown in panel A. The dashed line at A8 indicates the start of the decrease in the A signal and the increase in G signal.

(C) Representative sequencing chromatogram for the cellular pre-tRNAiMet sample from the trm6-504 strain. The dashed line at A4 indicates the start of the decrease in A signal and the increase in G signal. Experiments were repeated multiple times and virtually identical chromatograms were obtained.

3.2.4 Modulated polyadenylation activity with generic model substrates

We next examined in vitro whether the modulation of polyadenylation activity was specific for physiological TRAMP targets, or if TRAMP also polyadenylated simple model RNAs in a similar fashion. First, we tested a substrate consisting of a 16 bp duplex with a single nucleotide overhang at the 3’ end (Figure 3.9A). The protruding nucleotide was necessary to obtain appreciable levels of polyadenylation. On a 16 bp blunt end duplex, TRAMP showed exceedingly low activity (Figure 5.2).
Figure 3.9  Modulated polyadenylation activity with generic model substrates. 

(A) Polyadenylation of an RNA substrate consisting of a 16 bp duplex with 1 nt 3'-terminal overhang (100 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and 0.5 nM RNA) (upper panel). The asterisk marks the radiolabel. The 16 nt top strand contained a 3'-terminal 2',3'-dideoxy residue to prevent adenylation. Plots show rate constants at TRAMP and ATP saturation (\(k_{\text{max}}\), middle panel) and the apparent ATP affinity (\(K_{1/2,\text{ATP}}\), bottom panel) for individual adenylation steps. Values were determined from multiple reactions with increasing TRAMP and ATP concentrations (Figures 3.5-3.7). Error bars indicate the standard deviation.

(B) Polyadenylation of a 23 bp RNA duplex with 1 nt 3' overhang (top strand with 3'-terminal 2',3'-dideoxy residue). Gel and plots correspond to those in panel A.

(C) Polyadenylation of a 24 nt ssRNA substrate. Gel and plots correspond to those in panel A.

Adenylation rate constants (\(k_{\text{max}}\)) for the RNA duplex with the single nucleotide overhang displayed a clear peak, and ATP affinities for individual adenylation steps (\(K_{1/2,\text{ATP}}\))
showed a pronounced peak of low ATP affinity (Figure 3.9A), as seen for the tRNA_{i}^{Met} substrate (Figure 3.2). While both peaks were slightly shifted, compared to the tRNA_{i}^{Met} substrate, the sharp decrease in ATP affinity coincided again with the decrease in adenylation rate constants (Figure 3.9A). Extending the duplex to 23 bp had little effects on overall adenylation rate constants, presence of the characteristic peak in adenylation rate constants, and the corresponding decrease in ATP affinity, although the peaks were slightly shifted, compared to the 16 bp duplex (Figure 3.9B). The data obtained with these simplified model substrates clearly indicated that modulated polyadenylation activity is not restricted to physiological targets, but an inherent feature of TRAMP.

The slight shift of the peaks for polyadenylation rate constants and ATP affinities for the different substrates suggested potential effects of RNA structure on the modulation. It had been shown that RNA structure affected Mtr4p binding (Weir et al., 2010), but it was also possible that the modulation with the tested substrates was caused by Mtr4p-mediated duplex unwinding. To test whether the modulation of polyadenylation depended on duplex unwinding, we measured polyadenylation of a single stranded RNA. If modulation required unwinding, then the absence of duplexes would eliminate or drastically change the modulation. TRAMP displayed very low activity on a 17 nt ssRNA, likely due to weak affinity for the 17 nt ssRNA (Figure 3.10D, E). Affinities for initial adenylation steps apparently depend on both structure and length of the substrate (Figure 3.10).
Figure 3.10  Apparent substrate affinities ($K_{1/2}^{TRAMP}$) for ds and ssRNA model substrates
and reactions with the 17 nt ssRNA.
Affinities were determined as described for the tRNA$_{i}$Met (Figures 3.5, 3.6), but for the
substrates used in Figure 3.9. Cartoons indicate the substrates, the asterisks mark the radiolabel.
(A) Apparent substrate affinity for the RNA substrate consisting of a 16 bp duplex with a single nt 3’-
terminal overhang.
(B) Apparent substrate affinity for the 24 nt ssRNA substrate.
(C) Apparent substrate affinity for the 23 bp RNA duplex with a single nt 3’-terminal overhang. 12
instead of 10 steps are shown for consistency with Figure 3.9B.
(D) Representative time course of a polyadenylation reaction with the 17nt ssRNA (bottom strand of
the 16 bp-1 nt overhang duplex). The reaction was performed with 0.5 nM RNA, 100 nM TRAMP and
2 mM ATP-Mg$^{2+}$.
(E) Observed adenylation rate constant for the first adenylation step ($k_1$) plotted against wtTRAMP
concentration, for the 17 nt ssRNA substrate (filled circles), its duplex counterpart (16 bp - 1 nt
overhang, open circles) and the 24 nt ssRNA (filled triangles).

A 24 nt ssRNA was robustly adenylated by TRAMP (Figure 3.9C). Both adenylation
rate constants ($k_{max}$) and ATP affinities for the individual adenylation steps ($K_{1/2,ATP}$)
displayed the characteristic peaks, indicating modulated polyadenylation (Figure 3.9C).
Similar modulation was seen for other ssRNAs longer than 17 nt (data not shown). The
modulated polyadenylation activity on single stranded RNA indicates that the modulation is
not based on duplex unwinding by TRAMP. Notwithstanding, unwinding could still contribute to some extent to the observed slight influence of RNA secondary structure on TRAMP activity.

3.2.5 The modulation of polyadenylation activity depends on Mtr4p

If duplex unwinding was not causing the modulation of polyadenylation activity, did the helicase Mtr4p affect the modulation at all? To answer this question, we measured polyadenylation by a TRAMP complex without Mtr4p (Trf4p/Air2p). With the 16 bp duplex substrate described above, Trf4p/Air2p showed only low, unquantifiable levels of polyadenylation activity (Figure 3.11A, B). The 23 bp and the 24 nt ssRNA substrates were robustly polyadenylated (Figure 3.11C-H). With both substrates, Trf4p/Air2p produced longer poly(A) tails than TRAMP over comparable time frames (Figure 3.11A, C, F). Adenylation rate constants increased for the first two steps, but did not produce the characteristic peak seen with complete TRAMP (cf. Figures 3.9, 3.11). Similarly absent was the peak for ATP affinities (Figure 3.11E, H). The apparent substrate affinities showed a pattern similar to TRAMP, with the exception of the 2nd step for the 24 nt ssRNA (Figure 3.12). Together, these observations demonstrate that Trf4p/Air2p does not display the modulated polyadenylation activity seen with complete TRAMP. Mtr4p thus plays a critical role in the modulation of TRAMP activity. In a striking correlation with our observations, Mtr4p depletion in vivo causes hyperadenylation of TRAMP targets (Houseley and Tollervey, 2006).
Figure 3.11 Removal of Mtr4p abolishes modulation of polyadenylation activity.

(A) Polyadenylation of the 16 bp duplex substrate by Trf4p/Air2p (cf. Figure 3.9A). The reaction was performed with 0.5 nM 32P-labeled RNA, 100 nM Trf4p/Air2p and 2 mM ATP-Mg2+, under conditions equivalent to the reactions with TRAMP.

(B) Observed adenylation rate constant for the first step ($k_1$) plotted against Trf4p/Air2p concentration, for the 16 bp duplex substrate (filled circles), and the 24 nt single strand (open circles).

(C-E) Polyadenylation of the 23 bp RNA duplex with 1 nt 3' overhang (asterisk: radiolabel) by Trf4p/Air2p. Representative gel for reaction with 0.5 nM 32P-labeled RNA, 100 nM Trf4p/Air2p, 2 mM ATP-Mg2+ (panel C). Plots show rate constants at Trf4p/Air2p and ATP saturation ($k_{max}$, panel D) and the apparent ATP affinity ($K_{1/2, ATP}$, panel E) for individual adenylation steps. Values were determined from multiple reactions with increasing Trf4p/Air2p and ATP concentrations (as illustrated in Figures 3.5-3.7), error bars indicate the standard deviation. For reference, the dashed line marks A4. The y-
axis for the plot of apparent ATP affinities was broken to enable direct comparison of the identical reaction with wtTRAMP (Figure 3.9B).

**(F-H)** Polyadenylation of the 24 nt ssRNA substrate by Trf4p/Air2p. Representative gel for reaction with 0.5 nM 32P-labeled RNA, 100 nM Trf4p/Air2p, 2 mM ATP-Mg<sup>2+</sup> (panel F). Plots show rate constants at Trf4p/Air2p and ATP saturation ($k_{max}$, panel G) and the apparent ATP affinity ($K_{1/2}^{ATP}$, panel H) for individual adenylation steps.

**Figure 3.12** Apparent substrate affinities of Trf4p/Air2p ($K_{1/2}^{Trf4p/Air2p}$) for ds and ssRNA model substrates.

**(A)** Apparent substrate affinities of Trf4p/Air2p ($K_{1/2}^{Trf4p/Air2p}$) for the 23 bp duplex substrate. Affinities were determined as described for TRAMP and tRNA<sub>Met</sub> in Figure 3.5. The cartoon depicts the substrate, the asterisk marks the radiolabel.

**(B)** Apparent substrate affinities of Trf4p/Air2p ($K_{1/2}^{Trf4p/Air2p}$) for the 24 nt ssRNA substrate.

To illuminate how Mtr4p contributed to the modulation, we examined a TRAMP complex with a mutated Mtr4p (TRAMP<sup>Mtr4-20p</sup>). The Mtr4-20p mutation, located in the helicase motif VI, strongly decreases unwinding and RNA-stimulated ATPase activities of Mtr4p, but TRAMP<sup>Mtr4-20p</sup> retains polyadenylation activity (Wang et al., 2008). With all substrates tested, TRAMP<sup>Mtr4-20p</sup> generated longer poly(A) tails than wtTRAMP at comparable reaction times (Figure 3.13). With the 23 bp and the 24 nt ssRNA substrates, polyadenylation rate constants and ATP affinities showed only very broad peaks, which also lacked the coordination between changes in rate constants and ATP affinity seen with wtTRAMP (Figure 3.13B, C). With the 16 bp duplex substrate, no peaks in adenylation rate
Figure 3.13 Mutation of Mtr4p diminishes modulation of polyadenylation activity.

(A) Polyadenylation of the 16 bp duplex (1 nt 3' terminal overhang) by TRAMP<sup>Mtr4-20p</sup>. Representative gel for reaction with 0.5 nM <sup>32</sup>P-labeled RNA, 100 nM TRAMP<sup>Mtr4-20p</sup>, 2 mM ATP-Mg<sup>2+</sup> (upper panel). Plots show rate constants at Trf4p/Air2p and ATP saturation (<i>k</i><sub>max</sub>, middle panel) and the apparent ATP affinity (<i>K<sub>1/2</sub></i>, bottom panel) for individual adenylation steps. Values were determined from multiple reactions with increasing TRAMP and ATP concentrations (as illustrated in Figures 3.5-3.7), error bars indicate the standard deviation. For reference, the dashed line marks A<sub>4</sub>.

(B) Polyadenylation of the 23 bp RNA duplex (1 nt 3' overhang) by TRAMP<sup>Mtr4-20p</sup>. Gel and plots as in panel A. The inset shows the data with 10-fold magnification in the y-axis, to enable direct comparison of the identical reaction with wtTRAMP (Figure 3.9B).

(C) Polyadenylation of the 24 nt ssRNA substrate by TRAMP<sup>Mtr4-20p</sup>. Gel and plots as in panel A.
Figure 3.14  Apparent substrate affinities of TRAMP\textsuperscript{Mtr4-20p} for ds and ssRNA model substrates. 
(A) Apparent substrate affinities of TRAMP\textsuperscript{Mtr4-20p} (\(K_{1/2}^{\text{Mtr4-20p}}\)) for the 16 bp duplex substrate. Affinities were determined as described for wtTRAMP and tRNA\textsubscript{i}Met in Figure 3.5. The cartoon depicts the substrate, the asterisk marks the radiolabel. 
(B) Apparent substrate affinities of TRAMP\textsuperscript{Mtr4-20p} (\(K_{1/2}^{\text{Mtr4-20p}}\)) for the 23 bp duplex substrate. 
(C) Apparent substrate affinities of TRAMP\textsuperscript{Mtr4-20p} (\(K_{1/2}^{\text{Mtr4-20p}}\)) for the 24 nt ssRNA substrates.

Constants or ATP affinities were seen (Figure 3.13A). The effect of the mutation on substrate affinities was less pronounced than effects on rate constants and ATP affinities (Figure 3.14). The results collectively indicate that the Mtr4-20p mutation causes a precipitous loss in the capacity of TRAMP to modulate polyadenylation activity. This observation provides further evidence that Mtr4p plays a critical role in modulating TRAMP polyadenylation activity.

Moreover, the data reveal that the presence of Mtr4p in TRAMP alone is not sufficient to modulate polyadenylation. The modulation apparently requires Mtr4p with intact coordination between RNA and ATP binding sites, which is impaired in the Mtr4-20p mutant (Jackson et al., 2010; Wang et al., 2008; Weir et al., 2010).
3.2.6 The modulation of polyadenylation activity depends on the number of 3’-terminal adenylates

![Diagram](Figure 3.15 TRAMP adjusts polyadenylation activity based on the number of 3’-terminal adenylates. (A) Polyadenylation of a 24 nt ssRNA substrate with four terminal adenylates (filled symbols) by TRAMP. Plots show rate constants at TRAMP and ATP saturation ($k_{max}$, upper panel) and the apparent ATP affinity ($K_{1/2}^{ATP}$, bottom panel) for individual adenylation steps. For comparison, the identical substrate without the terminal adenylates is shown (open symbols, values identical to those in Figure 3.9C). The dashed lines mark $k_1$ and $k_5$, $A_1$ and $A_5$, the arrows emphasize the shift of the peaks for adenylation rate constants and apparent ATP affinities by four nucleotides. (B) Polyadenylation of a 24 nt ssRNA substrate with four consecutive adenylates 5 nt removed from the 3’-terminus (filled symbols). For comparison, values for the identical substrate without the terminal adenylates are shown (open symbols, as in panel A). Plots correspond to those in panel A.

Having implicated Mtr4p in the modulation of polyadenylation by TRAMP, we next asked how TRAMP determined at which steps to decrease adenylation rate constants and ATP affinities. A central point in this regard was whether TRAMP adjusted its activity only
for adenylates that it appended or also for adenylates already present in the RNA. To distinguish between these possibilities, we measured rate constants and ATP affinities for individual adenylation steps with a 24 nt ssRNA substrate containing four 3’-terminal adenosine residues (Figure 3.15A). Rate constants did not display the characteristic peak at A₄, but were remarkably similar to those measured for steps > 4 for the substrate without 3’-terminal adenylates (Figure 3.15A). ATP affinities showed a peak shifted by 4 positions, compared to the substrate without the 3’-terminal adenylates (Figure 3.15A, lower panel). This characteristic shift was not seen with a 24 ssRNA substrate containing four consecutive adenylates within the oligonucleotide (Figure 3.15B). The data demonstrate that TRAMP adjusts its activity based on the presence of a critical number of 3’-terminal adenylates, irrespective of whether or not they are appended by TRAMP.

3.2.7 Residues outside the helicase domain of Mtr4p participate in the detection of 3’-terminal adenylates

Since Mtr4p modulated polyadenylation, we next probed whether and how the 3’-terminal nucleotides were detected by Mtr4p during polyadenylation. A recent crystal structure of Mtr4p indicated a potential base recognition site, outside the helicase core (Weir et al., 2010, Figure 3.16A, B). The structure suggested that E947, which is highly conserved in Mtr4p orthologs, contacts adenosine-specific groups on the fourth base from the 5’ end of the RNA bound in the structure (Figure 3.16C).
**Figure 3.16 E947 in Mtr4p is critical for the modulation of polyadenylation.**

(A) Domain structure of Mtr4p (Weir et al., 2010). Domain names are shown. The blue bar represents E947.

(B) Crystal structure of Mtr4p in complex with ADP and 5 nt oligo(A). Molecule B from Weir et al. is shown. The domains are colored as in panel A. E947 is shown in blue and RNA in orange. The dashed circle marks the area magnified in panel C.

(C) Close up view of E947 and the 5 nt oligo(A). For clarity, only residues 945-951 and 1026-1036 in the helical bundle domain are shown (gray). E947 establishes a single H-bond with N6 of the 4th adenine from the 5' end (Weir et al., 2010).

(D) Polyadenylation of the 24 nt ssRNA substrate by TRAMP Mtr4p(E947A) (100 nM TRAMP Mtr4p(E947A), 2 mM ATP-Mg\(^{2+}\), 0.5 nM RNA).

(E) Rate constants at TRAMP Mtr4p(E947A) and ATP saturation (k_{max}, upper panel, filled circles), and apparent ATP affinity (K_{1/2}ATP, lower panel, filled squares) for individual adenylation steps. For comparison, values for wtTRAMP from Figure 3.9C are shown (open symbols). Values were determined from multiple independent reactions, error bars indicate the standard deviation.

(F) Apparent substrate affinity of TRAMP Mtr4p(E947A) for the 24 nt ssRNA substrate (filled diamonds). Values for wtTRAMP from Figure 3.10C are shown for comparison (open diamonds).
Reasoning that E947 might be involved in the identification of the critical number of 3’-terminal adenylates, we replaced E947 with an alanine. TRAMP with Mtr4p(E947A) (TRAMP_{Mtr4p(E947A)}) produced longer poly(A) tails than wtTRAMP over identical reactions times (Figure 3.16D). The peak in adenylation rate constants seen with TRAMP_{Mtr4p(E947A)}

![Diagram](image)

**Figure 3.17 Energetic differences of TRAMP_{Mtr4-20p} and TRAMP_{Mtr4(E947A)} compared to wtTRAMP for each adenylation step.** Effects of the Mtr4-20p mutation (M540I) (A) and the Mtr4p E947A mutation (B) on observed free activation enthalpies for observed adenylation rate constants adenylation (upper panels), on the free energies of functional ATP affinities (middle panels) and apparent substrate affinities (lower panels) for individual adenylation steps. The effect is expressed as difference in the respective free activation enthalpies and free energies, e.g., $\Delta \Delta G^\ddagger = \Delta G^\ddagger_{(wtTRAMP)} - \Delta G^\ddagger_{(mtTRAMP)}$. Free activation enthalpies were calculated according to $\Delta G^\ddagger = -RT \ln(hk_T/k_B T)$ (R: gas constant, T: temperature, h: Planck constant, k: rate constants determined in Figure 3.9C (wt), Figure 3.13C (TRAMP_{Mtr4-20p}), or Figure 3.16E (TRAMP_{Mtr4(E947A)}), k_B: Boltzmann constant). Free energies for functional ATP affinities were calculated according to $\Delta G^\ddagger = -RT \ln(1/K^{1/2}_{ATP})$, using the ATP affinities ($K^{1/2}_{ATP}$) determined in Figure 3.9C (wt), Figure 3.13C (TRAMP_{Mtr4-20p}), or Figure 3.16E (TRAMP_{Mtr4(E947A)}). Free energies for substrate affinities were calculated according to $\Delta G^\ddagger = -RT \ln(1/K^{1/2}_{TRAMP})$, using the substrate affinities ($K^{1/2}_{TRAMP}$) determined in Figure 3.10C (wt), Figure 3.14C (TRAMP_{Mtr4-220p}), or Figure 3.16F (TRAMP_{Mtr4(E947A)}). The arrows on the right indicate how these energy differences correspond to slower/faster rate constants and weaker/tighter ATP binding, thus quantitatively describing effects of the Mtr4p mutations on the reaction parameters for each adenylation step.
was significantly broader than with wtTRAMP (Figure 3.16E). No clear peak at all was seen for ATP affinities, which were also much lower for later steps (7-10) than for wtTRAMP (Figure 3.16E). In addition, initial substrate affinity was also impaired in the mutant (Figure 3.16F). Thus, TRAMP\textsuperscript{Mtr4p(E947A)} markedly diminished the modulation of polyadenylation, similar to the Mtr4-20p mutation in TRAMP\textsuperscript{Mtr4-20p} (Figure 3.17). We conclude that E947 is important for modulating polyadenylation, consistent with a scenario where Mtr4p directly binds the 3’-terminal nucleotides, and upon detection of 3’-terminal adenylates, alters the polyadenylation activity of Trf4p.

3.2.8 Generation of short poly(A) tails involves multiple cycles of TRAMP binding and dissociation

To further understand how the polyadenylation activity was modulated, it was important to examine whether multiple binding and dissociation events were required until 4 adenylates were added. To answer this question, we determined the processivity of TRAMP for individual adenylation steps in the reaction with the 24 nt ssRNA (Figure 3.18, see Appendix 3 for details). The processivity is the probability of TRAMP adding the next adenosine vs. dissociating from the substrate (Figure 3.18A). This probability is directly related to the average number of steps per binding event (Ali and Lohman, 1997). When re-binding of dissociated TRAMP was precluded by the addition of excess scavenger RNA (73 nt ssRNA of unrelated sequence, see Table 10.1 for sequence) (Figure 3.18B), further polyadenylation, although limited, reflected processivity of the reaction (Figure 3.18C, D).

We developed a computer routine to calculate processivity from the difference in poly(A) distribution before and after scavenger challenge (Figure 3.18, Appendix 3). Plots of
processivity vs. number of added adenylates revealed a steady increase in processivity until \( P = 0.64 \pm 0.10 \) at \( A_4 \), followed by a slight decrease to \( P = 0.40 \pm 0.03 \) at \( A_{10} \) (Figure 3.19A). For the first step, TRAMP dissociates roughly four times faster than it adds the adenosine, i.e. about 5 binding events are necessary to add the first adenosine (\( P = 0.21 \pm 0.12 \)). For subsequent steps, dissociation and adenylation are roughly equally fast, i.e., TRAMP adds roughly 2 nucleotides per binding event (Figure 3.19A). The data show that TRAMP undergoes multiple binding and dissociation cycles to append 4 to 5 adenylates.

Figure 3.18 Measurement of TRAMP processivity for the polyadenylation reaction. (A) Reaction scheme illustrating the principle of processivity \( (P_{1\ldots n}) \) for individual adenylation steps \((T:\) TRAMP, \( A_{0\ldots n}\): adenylated RNA species, \( TA_{0\ldots n}\): TRAMP bound to the respective adenylated species, \( k_{i\ldots n}\): adenylation rate constant for individual steps, \( k_{diss\ldots n}\): dissociation rate constant for individual steps). For corresponding equations used for the determination of processivities for individual steps, see Appendix 3.
(B) Reaction diagram for processivity measurements of TRAMP-catalyzed polyadenylation. Polyadenylation reactions were allowed to proceed for a defined time (t₁). Then, a scavenger RNA was added to prevent re-binding of TRAMP (73 nt ssRNA with sequence unrelated to substrate RNA). The scavenger was verified to completely sequester TRAMP at the concentrations used in the reaction (data not shown). After scavenger addition, the reaction was allowed to proceed for a defined time (t₂), until the distribution of polyadenylated species no longer changed (10 min following scavenger addition, data not shown). Samples were then applied to denaturing PAGE to resolve individual adenylation steps.

(C) Representative denaturing PAGE for processivity measurements. Polyadenylation reactions were performed with 0.5 nM 24 nt ssRNA substrate, 150 nM wtTRAMP and 2 mM ATP-Mg²⁺. Polyadenylation was allowed to proceed for t₁ = 2 min and t₁ = 8 min, respectively (lanes 1 and 4), after which times the scavenger RNA was added (73 nt ssRNA, 10 μM final concentration). After scavenger addition, aliquots were removed from the reaction at t₂ = 2 min (lanes 2, 5) and t₂ = 4 min (lanes 3, 6). The fraction of each adenylated RNA was quantified, yielding distributions of polyadenylated species at a given reaction time before (t₁) and after (t₂) scavenger addition (plotted in panel C).

(D) Shift in the distribution of polyadenylated RNA species after scavenger addition. Relative abundance of individual species (Aᵢ / Σ (A₀…Aᵢ), i = 1…n) was plotted vs. poly(A) length before (upper panel) and after scavenger addition (lower panel). The shift in the peak position of the distribution before and after scavenger addition is marked with the arrow. Processivity for each step is calculated by comparing the distributions before and after scavenger addition, as described in Appendix 3.

![Figure 3.19 Processivity of polyadenylation and the effect of Mtr4p on actual adenylation and enzyme dissociation.](image)

(A) Processivity of TRAMP for individual adenylation steps with the 24 nt ssRNA substrate (0.5 nM RNA, 150 nM TRAMP, 2 mM ATP-Mg²⁺). Reactions were performed as shown in Figure 3.18, and processivity was calculated from the distributions of polyadenylated species according to Appendix 3. The average number of steps (N), shown at the right, corresponds to the processivity according to: \( P = (N-1)/N \) (Ali and Lohman, 1997). The dotted line marks \( P = 0.5 \), \( N = 2 \). Processivity values are the average from multiple independent measurements with a range of reaction time, the error bars mark one standard deviation.

(B) Processivity of Trf4p/Air2p for individual adenylation steps of the 24 nt ssRNA substrate (0.5 nM RNA, 150 nM Trf4p/Air2p, 2 mM ATP-Mg²⁺).
Actual adenylation rate constants \( (k_f^n, \text{panel C}) \) and dissociation rate constants \( (k_{\text{diss}}^n, \text{panel D}) \) of TRAMP (filled circles) and Trf4p/Air2p (open circles) for individual adenylation steps with the 24 nt ssRNA substrate. Rate constants were calculated according to Equation A7 with \( k_f^n + k_{\text{diss}}^n = k_{\max}^n \) (Ali and Lohman, 1997). Values shown were calculated from the data in Figures 3.9C, 3.11G and panels A, B. Error bars mark one corresponding standard deviation.

### 3.2.9 Mtr4p modulates Trf4p activity through multiple, energetically small effects

We next examined the effects of Mtr4p on TRAMP processivity. TRAMP without Mtr4p (Tr4p/Air2p) showed lower processivity than wtTRAMP for the first four steps. Subsequent steps displayed a slightly higher processivity than wtTRAMP (Figure 3.19B). To understand the influence of Mtr4p on a more quantitative level, we calculated forward and dissociation rate constants for each adenylation step for TRAMP with and without Mtr4p (Figure 3.19C, D). For steps 1 – 3, Mtr4p enhances polyadenylation rate constants, and then slows these rate constants for subsequent steps (Figure 3.19C). In addition, Mtr4p enhances TRAMP dissociation for the first step. For subsequent steps, Mtr4p decreases dissociation rate constants, thus prolonging the time TRAMP remains bound to the RNA (Figure 3.19D).

To visualize the multifaceted, coordinated effects of Mtr4p on the Trf4p activity, we calculated energetic contributions of Mtr4p to adenosine addition, TRAMP dissociation and ATP affinity for individual adenylation steps (Figure 3.20A, B). Compared to the reaction without Mtr4p, the helicase enhances adenylation rate constants and promotes tighter ATP binding for the first two steps (Figure 3.20C). For steps 4 and higher Mtr4p slows adenylation rate constants and weakens ATP binding (Figure 3.20C). Mtr4p slows TRAMP dissociation from the RNA, except for the first step (Figure 3.20C). In energetic terms, the impact of Mtr4p is greatest on ATP affinities and adenylation rate constants. In general, however, Mtr4p imparts rather small changes on the individual rate constants. Yet, numerous small effects multiply over many steps and thus significantly alter the polyadenylation
pattern, compared to the reaction without or with impaired Mtr4p. The coordination between changes in rate constants and changes in ATP affinity provides additional synergy to favor a temporal accumulation of short poly(A) tails (Figure 3.20C).

Figure 3.20 Energetic effects of Mtr4p on various parameters in TRAMP-mediated polyadenylation reaction.

(A) Free activation enthalpies ($\Delta G^\dagger$) for adenylation (upper panels) and dissociation (middle panels), and the free energy for ATP affinities ($\Delta G^\circ$, lower panels) for individual adenylation steps for TRAMP (left panels) and Trf4p/Air2p (right panels), measured for the 24 nt ssRNA substrate. Free activation enthalpies were calculated according to $\Delta G^\dagger = -RT \cdot \ln(hk_b/k_bT)$ (R: gas constant, T: temperature, h: Planck constant, $k_b$: Boltzmann constant). Free energies for functional ATP affinities were calculated according to $\Delta G^\circ = -RT \cdot \ln(1/K_{1/2}^{\text{ATP}})$, using the ATP affinities ($K_{1/2}^{\text{ATP}}$) determined in Figure 3.9C (TRAMP) and Figure 3.11H (Trf4p/Air2p).

(B) Mtr4p effects on free activation enthalpies for adenylation (upper panel) and dissociation (middle panel), and on the free energies of functional ATP affinities (lower panels) for individual adenylation steps. The effect is expressed as difference in the respective free activation enthalpies and free energies shown in panel A, e.g., $\Delta \Delta G^\dagger = \Delta G^\dagger(\text{TRAMP}) - \Delta G^\dagger(\text{Trf4p/Air2p})$. The arrows on the right show how energy differences correspond to slower/faster rate constants and weaker/tighter ATP binding for each adenylation step.
3.3 Discussion

3.3.1 A key role for Mtr4p in the regulation of poly(A) tail lengths for RNAs processed by TRAMP

In this study, we have shown that the RNA helicase Mtr4p regulates polyadenylation in the TRAMP complex. By modulating individual adenylation steps, Mtr4p facilitates a temporary accumulation of RNAs with short poly(A) tails of only ~4 adenylates \textit{in vitro}. Strikingly, TRAMP targets \textit{in vivo} accumulate similarly short poly(A) tails. We demonstrate that hypomethylated pre-tRNA$_{\text{Met}}$, a prototypical TRAMP target, accumulates poly(A) tails with roughly 4 nucleotides (Figure 3.8). Previous reports showed similarly short poly(A) tails on other TRAMP targets (Grzechnik and Kufel, 2008; Lebreton \textit{et al.}, 2008), and a recent analysis of a wide range of TRAMP targets found a distribution of poly(A) tails with a pronounced peak at 3 to 5 nucleotides (Wlotzka \textit{et al.}, 2011). The remarkable agreement between these observations made \textit{in vivo} and our \textit{in vitro} data suggests a physiological role of Mtr4p in the control of the lengths of poly(A) tails produced by TRAMP. This notion further concurs with data showing hyperadenylation upon Mtr4p depletion \textit{in vivo} (Houseley and Tollervey, 2006). Accordingly, removal of Mtr4p \textit{in vitro} abolishes modulation and eliminates poly(A) tail length restriction (Figure 3.11). The striking correlation between several lines of experiments \textit{in vivo} and \textit{in vitro} is consistent with a pivotal, physiological role of Mtr4p in the regulation of polyadenylation by TRAMP. This role is in addition to the previously shown function of Mtr4p as an exosome co-factor (Houseley and Tollervey, 2008; LaCava \textit{et al.}, 2005). Defects in the function of Mtr4p as an exosome co-factor might also
contribute to changes in poly(A) length of TRAMP targets seen in vivo with functionally impaired Mtr4p.

The multiple functional roles of Mtr4p mark this RNA helicase as a central player in the control of critical steps of the TRAMP-exosome machinery. Regulation of Mtr4p could simultaneously affect multiple steps in TRAMP/exosome-mediated RNA decay and processing. The Mtr4p-mediated restriction of poly(A) tail lengths might prevent RNAs processed by TRAMP from binding to poly(A) binding proteins, the S. cerevisiae form of which (Pab1p) binds 12 adenylates per protein molecule (Sachs et al., 1987). In addition, the short poly(A) tails appended by TRAMP may serve as a specific signal on the RNA for subsequent recognition by the exosome (Bernstein et al., 2010).

3.3.2 A new type of function for an RNA helicase: control of another enzyme in response to features in the RNA

Mtr4p modulates the polyadenylation activity of Trf4p for a wide range of simple model substrates (Figures 3.2-3.7, 3.9, 3.11). These results indicate that the modulation by Mtr4p is an inherent feature of TRAMP, and not conferred by certain substrates. The regulation of Trf4p by Mtr4p represents a new type of function for an RNA helicase; the modulation of another enzyme in response to features in the RNA, here the presence or emergence of a certain number of 3’ adenylates. The modulation seen with ssRNA shows that this function of Mtr4p does not depend on duplex unwinding (Figure 3.9). Modulation of Trf4p by Mtr4p therefore differs from unwinding-based effects of RNA helicases on viral RNA polymerases and on RNA degradation by bacterial and mitochondrial degradosomes (Borowski et al., 2010; Carpousis et al., 2009; Piccininni et al., 2002).
Notwithstanding, RNA structure slightly influences the degree by which Mtr4p modulates Trf4p (Figure 3.9). Probably, this effect is mainly caused by the impact of RNA structure on TRAMP binding, as reflected by TRAMP affinities for RNA (Figures 3.5, 3.10). Effects of RNA structure on TRAMP binding are consistent with the impact of secondary structure on RNA binding by Mtr4p, as seen previously by others (Weir et al., 2010). Nevertheless, duplex unwinding might still contribute slightly to the observed modulation of polyadenylation by Mtr4p.

Although the modulation of Trf4p by Mtr4p does not depend on duplex unwinding, helicase activity is most likely important after polyadenylation, to stimulate exosome function on structured substrates (LaCava et al., 2005; Wang et al., 2008). RNA degradation by the exosome is thought to require ssRNA much longer than the short poly(A) tails found on TRAMP targets (Bonneau et al., 2009), and Mtr4p-catalyzed duplex unwinding may be necessary to generate sufficiently long stretches of ssRNA (Anderson and Wang, 2009).

The ability of Mtr4p to modulate Trf4p activity through a series of energetically rather minor adjustments (Figure 3.20C) is a notable and new paradigm for a regulatory function by an RNA helicase. It is interesting to speculate about the benefits of this mode of regulation, compared to a total shutoff of polyadenylation after a set number of adenylation steps. Numerous small adjustments might provide TRAMP with flexibility to accommodate diverse substrates (Wlotzka et al., 2011), and to coordinate polyadenylation with subsequent processing steps (Callahan and Butler, 2010).

### 3.3.3 Direct interrogation of 3’-terminal bases by Mtr4p

How can we physically imagine Mtr4p exerting its modulatory effects in the context of TRAMP? Guided by a recent crystal structure of Mtr4p, which suggests contacts between
protein and nucleobases via residues located outside the helicase core (Weir et al., 2010), we show that at least one of these residues, E947, is critical for the modulation. Mutation of E947 markedly diminishes modulation by Mtr4p (Figure 3.16). This convergence of structural and biochemical data suggests that Mtr4p directly reads out the sequence at the 3’-terminus in the context of TRAMP, most likely by binding the 3’-terminus in a conformation similar or identical to that seen in the crystal structure (Weir et al., 2010). This notion is supported by the diminished modulation seen with the Mtr4-20p mutation (Figure 3.13). This mutation impairs the coupling between ATP and RNA binding, and thus interferes with ATP-dependent contacts to the RNA backbone that are established by residues in the helicase core (Weir et al., 2010).

Although Mtr4p starts to modulate Trf4p as soon as a single 3’-terminal adenosine is present, the effects increase until four to five 3’-terminal adenylates are detected (Figure 3.20C). This observation suggests that Mtr4p binding to four to five adenylates is needed for the restriction of the poly(A) tail length. Binding of Mtr4p to 4 to 5 adenylates is consistent with the crystal structure (Weir et al., 2010). In addition, the binding site of isolated Mtr4p was recently shown to encompass approximately 5 nucleotides (Bernstein et al., 2010). Mtr4p was also shown to bind RNAs with adenylates tighter than other sequences (Bernstein et al., 2010; Bernstein et al., 2008). In TRAMP, this increased affinity might contribute to the Mtr4p-induced slowing of TRAMP dissociation from terminal adenylates (Figures 3.19D, 20C).

Multiple lines of structural and biochemical evidence thus coalesce around a model where Mtr4p binding to roughly four 3’-terminal adenylates restricts further adenylation by TRAMP. Addition of four adenylates by TRAMP involves multiple binding and dissociation
events (Figure 3.19A), which provide repeated opportunities for Mtr4p to interrogate the 3’ terminal bases and to adjust the Trf4p activity. Which molecular events take place during this adjustment will be a central question in the further investigation of TRAMP function.
4.1 Introduction

RNA duplex unwinding activity has not been carefully examined for any Ski2-like helicase (Section 1.4). Biochemical characterization of the S. cerevisiae Ski2-like protein Mtr4p has demonstrated RNA-stimulated ATPase activity and ATP-dependent RNA duplex unwinding activity (Wang et al., 2008; Bernstein et al., 2008) (Section 2.1.4). Such unwinding activity in the TRAMP complex has long been assumed, due to the plethora of RNA secondary structures in exosome substrates that likely resist degradation (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005; Liu et al., 2006). It is not known however, whether and how Mtr4p unwinds RNA duplexes in the presence of Trf4p and Air2p, as RNA helicases can be inhibited as well as stimulated by cofactors (Section 1.4). The TRAMP complex represents an excellent opportunity for understanding the activity of an RNA helicase in a physiological complex.

The coexistence of the polymerase Trf4p and the helicase Mtr4p in the TRAMP complex has intrigued researchers for years (LaCava et al., 2005). Chapter 3 showed that polyadenylation activity is intrinsically modulated in the TRAMP complex. Mtr4p orchestrates a series of adjustments in the activity of Trf4p to ensure a temporary accumulation of short oligo(A) tails of ~4 nt. The modulation of Trf4p activity by Mtr4p occurs on both structured and unstructured substrates and is thus independent of unwinding (Figure 3.9). Polyadenylation of structured substrates could foreseeably affect unwinding by
Mtr4p. It is not clear, however, whether and how polyadenylation by Trf4p and unwinding by Mtr4p is coordinated for a given substrate.

In this chapter, I report characterization of the unwinding activity of Mtr4p alone and in the TRAMP complex and demonstrate tight coupling between polyadenylation and unwinding in the TRAMP complex.

4.2 Results

4.2.1 Trf4p/Air2p in the TRAMP complex stimulates the unwinding activity of Mtr4p

To quantitatively assess how the helicase activity of Mtr4p is affected in the TRAMP complex, we measured unwinding rate constants of recombinant Mtr4p and TRAMP. We used a well-characterized model substrate containing a 16 bp duplex and a 3’ single-stranded extension of 25 nt (Figure 4.1A). This substrate had been previously employed for measuring unwinding by Mtr4p (Wang et al., 2008). Unwinding reactions were performed under pre-steady state conditions (enzyme in excess over substrate) in the presence of dATP to eliminate polyadenylation by Trf4p (Figure 4.1A, Figure 4.2). At identical enzyme concentrations, TRAMP unwound the duplex significantly faster than Mtr4p alone (Figure 4.1A, B). Unwinding rate constants ($k_{\text{unw}}$, see Section 4.2.3) were then determined for a range of TRAMP and Mtr4p concentrations (Figure 4.1C). At enzyme saturation, unwinding rate constants for TRAMP were roughly 8-fold higher than for Mtr4p (Figure 4.1C). No significant difference in the functional substrate affinities between TRAMP and Mtr4p was seen with this substrate (Figure 4.1C). These data demonstrate that the unwinding activity of Mtr4p is enhanced by Trf4p/Air2p in the TRAMP complex.
Figure 4.1 Unwinding activity of Mtr4p is stimulated in the TRAMP complex.
(A) Native PAGE showing unwinding of a 16 bp duplex containing 25 nt single-stranded region at the 3'-terminus by Mtr4p (left panel) and TRAMP (right panel). Reactions were performed with 0.5 nM 32P-labeled duplex (asterisk marks the radiolabel), 200 nM Mtr4p or TRAMP and 2 mM equimolar dATP-Mg2+. Aliquots were removed after 1, 3, 10, 20, 60 min from the reaction with Mtr4p (left panel), and after 0.5, 1, 3, 10, 20 min from the reaction with TRAMP (right panel).
(B) Time traces for unwinding reactions with Mtr4p (left panel) and TRAMP (right panel). Reactions as shown in panel A were quantified and the fraction of single strand was plotted over time. Data were averaged for three independent repeats and error bars indicate the standard deviation. Curves represent best fits to first-order rate law. For Mtr4p, \(A_{\text{amp unw}} = 0.846 \pm 0.035, k_{\text{obs, unw}} = 0.06 \pm 0.01 \text{ min}^{-1}\). For TRAMP, \(A_{\text{amp unw}} = 0.885 \pm 0.026, k_{\text{obs, unw}} = 0.59 \pm 0.06 \text{ min}^{-1}\).
(C) Enzyme titrations for unwinding of the duplex by Mtr4p, wtTRAMP and TRAMPTrf4-236p. Unwinding rate constants were plotted over concentrations of Mtr4p (open circles), wtTRAMP (filled black circles) and TRAMPTrf4-236p (filled gray circles). Rate constants were determined from multiple reactions (panels A, B) at 0.5 nM RNA, 2 mM dATP-Mg2+ and the enzyme concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherme \(k_{\text{unw}} = k_{\text{max, E}} [E] / ([E] + K_{1/2, E})\), where [E] represents enzyme concentration. For Mtr4p, \(k_{\text{max, Mtr4p}} = 0.090 \pm 0.003 \text{ min}^{-1}, K_{1/2, \text{Mtr4p}} = 105 \pm 36 \text{ nM}\). For wtTRAMP, \(k_{\text{max, TR}} = 0.84 \pm 0.10 \text{ min}^{-1}, K_{1/2, \text{TR}} = 94 \pm 31 \text{ nM}\). For TRAMPTrf4-236p, \(k_{\text{max, TR}} = 0.76 \pm 0.06 \text{ min}^{-1}, K_{1/2, \text{TR}} = 105 \pm 20 \text{ nM}\).
(D) dATP titrations for unwinding of the duplex by Mtr4p, wtTRAMP and TRAMPTrf4-236p. Unwinding rate constants were determined from multiple reactions (panels A, B) at 800 nM Mtr4p (open circles), 300 nM wtTRAMP (filled black circles) or TRAMPTrf4-236p (filled gray circles) and the dATP concentrations indicated. Error bars represent the standard deviation. For Mtr4p, the curve represents best fit to equation \(k_{\text{unw}} = k_{\text{max, dATP}} [\text{dATP}] / ([\text{dATP}] + (K_{1/2, \text{dATP}}))\), where \(k_{\text{max, dATP}} = 0.11 \text{ min}^{-1}\) (fixed for fitting according to results with ATP, Figure 4.2B). \(K_{1/2, \text{dATP}} = 2.08 \pm 0.27 \text{ mM}\). For wtTRAMP and TRAMPTrf4-236p, the curves represent best fits to equation \(k_{\text{unw}} = k_{\text{max, dATP}} [\text{dATP}]^2 / ([\text{dATP}]^2 + (K_{1/2,}...
For wtTRAMP, $k_{\text{max}, \text{dATP}} = 0.54 \pm 0.02 \text{ min}^{-1}$; $K_{1/2, \text{dATP}} = 0.74 \pm 0.04 \text{ mM}$. For TRAMP<sub>Trf4-236p</sub>, $k_{\text{max}, \text{dATP}} = 0.44 \pm 0.04 \text{ min}^{-1}$, $K_{1/2, \text{dATP}} = 0.58 \pm 0.12 \text{ mM}$.

**Figure 4.2** dATP allows unwinding without polyadenylation.  
(A) Denaturing PAGE showing TRAMP-catalyzed adenylation reactions with dATP and ATP. Reactions were performed with 0.5 nM 24 nt ssRNA, 200 nM TRAMP and 2 mM dATP or ATP-Mg<sup>2+</sup>, or 50 nM TRAMP and 2 mM ATP-Mg<sup>2+</sup>. Aliquots were removed after 0.5, 1, 3, 10, 20 min from the reaction with dATP, and after 3 min from the reaction with ATP. An unknown species marked by the asterisk emerged in the reaction with dATP, apparently smaller than the substrate with one AMP residue added.  
(B) ATP and dATP titrations for unwinding of the 16 bp duplex with 25 nt overhang by Mtr4p. Unwinding rate constants were determined from multiple reactions (Figure 4.1A, B) at 800 nM Mtr4p and the ATP and dATP concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to equation $k_{\text{unw}} = k_{\text{max}, \text{dATP}} [(\text{dATP})] / ([\text{dATP}] + K_{1/2, \text{dATP}})$. With ATP, $k_{\text{max}, \text{ATP}} = 0.11 \pm 0.03 \text{ min}^{-1}$, $K_{1/2, \text{ATP}} = 1.74 \pm 0.75 \text{ mM}$. With dATP, $k_{\text{max}, \text{dATP}} = 0.44 \pm 0.04 \text{ min}^{-1}$, $K_{1/2, \text{dATP}} = 0.58 \pm 0.12 \text{ mM}$, as shown in Figure 4.1D.  
(C) ATP and dATP titrations for unwinding of the duplex by TRAMP<sub>Trf4-236p</sub>. Unwinding rate constants were determined from multiple reactions at 300 nM TRAMP<sub>Trf4-236p</sub> and the ATP (filled circles) and dATP (open circles) concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to equation $k_{\text{unw}} = k_{\text{max, dATP}} [(\text{dATP})]^2 / ([[\text{dATP}}]^2 + (K_{1/2, \text{dATP}})^2)$. With ATP, $k_{\text{max}, \text{ATP}} = 0.43 \pm 0.02 \text{ min}^{-1}$, $K_{1/2, \text{ATP}} = 0.64 \pm 0.05 \text{ mM}$. With dATP, $k_{\text{max}, \text{dATP}} = 0.44 \pm 0.04 \text{ min}^{-1}$, $K_{1/2, \text{dATP}} = 0.58 \pm 0.12 \text{ mM}$, as shown in Figure 4.1D.

To illuminate how the unwinding activity of Mtr4p is stimulated in the TRAMP complex, we next examined the dependence of unwinding rate constants for Mtr4p and TRAMP on dATP concentration (Figure 4.1D). For Mtr4p, the unwinding rate constant was roughly half-saturated under 2 mM dATP (Figure 4.1D). The use of significantly higher concentrations of dATP-Mg<sup>2+</sup> was experimentally unfeasible due to inhibition of unwinding..
by free Mg$^{2+}$ introduced. The functional affinities of Mtr4p for dATP and ATP ($K_{1/2,(d)ATP}$) did not significantly differ (Figure 4.2B).

To further verify that the use of dATP instead of ATP only inhibited polyadenylation and not unwinding by TRAMP, we generated a Trf4p mutant (Trf4-236p, D236A,D238A) that does not display polyadenylation activity in the presence of ATP (Vanacova et al., 2005) (data not shown). Indeed, TRAMP$^{Trf4-236p}$ displayed virtually identical functional affinities for dATP and ATP (Figure 4.2C). TRAMP$^{Trf4-236p}$ showed unwinding rate constants, functional substrate affinities, and functional dATP affinities highly similar to those seen with wtTRAMP (Figure 4.1C, D), indicating that the polyadenylation activity of Trf4p is not required to promote unwinding by Mtr4p.

However, both wtTRAMP and TRAMP$^{Trf4-236p}$ displayed notably higher affinity for (d)ATP than Mtr4p alone ($K_{1/2,dATP} = 0.74 \pm 0.04$ mM for wtTRAMP; $K_{1/2,dATP} = 0.58 \pm 0.12$ mM for TRAMP$^{Trf4-236p}$) (Figures 4.1D, 4.2B, C). The increase in (d)ATP affinity in TRAMP partially explains the stimulation of Mtr4p-mediated unwinding in the presence of Trf4p/Air2p, leaving possible a direct effect on unwinding rate constants. A Hill coefficient of $n = 1.9 \pm 0.2$ was determined for wtTRAMP and TRAMP$^{Trf4-236p}$ (Figure 4.1D) ($n = 1.50 \pm 0.04$ for Mtr4p). This observation is consistent with a scenario where efficient unwinding by TRAMP requires (d)ATP binding to both the helicase and the polymerase, even though the polymerase activity of Trf4p is not required for the stimulation of the unwinding activity of Mtr4p.
4.2.2 Mtr4p displays polar unwinding but is sensitive to duplex length/stability

To further investigate the impact of Trf4p/Air2p on the unwinding activity of Mtr4p, we extended the 16 bp duplex containing 25 nt overhang by 20 bp (36 bp - 25 nt overhang) and measured unwinding by Mtr4p and TRAMP (Figure 4.3A). The reaction with Mtr4p showed ~20% of the duplex unwound after one hr. In the presence of TRAMP, ~70% of the duplex was unwound after 20 min. Thus, unlike DEAD-box proteins, the Ski2-like helicase Mtr4p is capable of unwinding RNA duplexes as long as 36 bp; Trf4p/Air2p in the TRAMP complex significantly enhance the unwinding capacity.
Figure 4.3 Mtr4p and TRAMP-mediated unwinding and annealing of a 36 bp duplex.

(A) Unwinding of a 36 bp duplex containing 25 nt single-stranded region at the 3'-terminus by Mtr4p (left panel) and TRAMP (right panel). Reactions were performed with 0.5 nM $^{32}$P-labeled duplex (asterisk marks the radiolabel), 200 nM Mtr4p or TRAMP and 2 mM equimolar dATP-Mg$^{2+}$. Aliquots were removed after 1, 3, 10, 20, 60 min from the reaction with Mtr4p (left panel), and after 1, 3, 6, 10, 20 min from the reaction with TRAMP (right panel).

(B) Annealing of the duplex by Mtr4p (left panel) and TRAMP (right panel). Reactions were performed with 0.5 nM denatured $^{32}$P-labeled duplex (asterisk marks the radiolabel), 200 nM Mtr4p or TRAMP and 2 mM equimolar dATP-Mg$^{2+}$. Aliquots were removed after 1, 3, 10, 20, 60 min from the reaction with Mtr4p (left panel), and after 1, 3, 6, 10, 20 min from the reaction with TRAMP (right panel).

(C) Convergence of unwinding and annealing traces of the duplex with Mtr4p (left panel) and TRAMP (right panel). Reactions as shown in panels A, B were quantified and the fraction of single strand was plotted over time. Data were averaged for three independent repeats and error bars indicate the standard deviation. Curves represent best fits to first-order rate law. For Mtr4p, $Amp_{unw} = 0.224 \pm 0.102$, $k_{obs, unw} = 0.02 \pm 0.02 \text{ min}^{-1}$; $Amp_{ann} = 0.697 \pm 0.152$, $k_{obs, unw} = 0.02 \pm 0.01 \text{ min}^{-1}$. For TRAMP, $Amp_{unw} = 0.733 \pm 0.057$, $k_{obs, unw} = 0.22 \pm 0.05 \text{ min}^{-1}$; $Amp_{ann} = 0.352 \pm 0.065$, $k_{obs, ann} = 0.13 \pm 0.05 \text{ min}^{-1}$.

The unwinding rate constant ($k_{unw}$) for TRAMP reached a $k_{max, TR} = 0.15 \pm 0.03 \text{ min}^{-1}$ at enzyme saturation (data not shown), corresponding to only $\sim 1/6$ of the rate constant measured with the 16 bp duplex (Figure 4.1C, $k_{max, TR} = 0.84 \pm 0.10 \text{ min}^{-1}$). We were not able to perform a reliable titration of Mtr4p due to its low activity for the 36 bp duplex (Figure 4.3A, C). Still, the data suggest that unwinding rate constants for TRAMP are sensitive to duplex length/stability, unlike viral RNA helicases of the NS3/NPH-II family that show only small or no effects (Jankowsky et al., 2000; Pang et al., 2002).

We noticed that duplex unwinding did not reach completion for either Mtr4p or TRAMP. The low unwinding amplitude raised the question whether Mtr4p and TRAMP also catalyze the reverse reaction, strand annealing. Strand annealing activity has been reported for a number of DEAD-box helicases (Rossler et al., 2001; Chamot et al., 2005; Yang and Jankowsky, 2005; Halls et al., 2007), but has not been shown for Ski2-like helicases. In order to test whether Mtr4p promotes strand annealing, we performed reactions under enzyme and dATP concentrations identical to those in the unwinding reactions, but with the denatured pair of single strands instead of the duplex. Indeed, $\sim 50\%$ of the ssRNA was converted to
duplex after one hr of incubation with Mtr4p (Figure 4.3B), significantly more than without Mtr4p (data not shown). Annealing of the duplex was also observed for TRAMP (Figure 4.3B). Together, our results show that both Mtr4p and TRAMP facilitate duplex annealing.

**Figure 4.4** Annealing activity of Mtr4p and TRAMP in formation of the 16 bp duplex containing 25 nt overhang.

(A) Annealing of the duplex by Mtr4p (left panel) and TRAMP (right panel). Reactions were performed with 0.5 nM denatured \(^{32}\)P-labeled duplex (asterisk marks the radiolabel), 200 nM Mtr4p or TRAMP and 2 mM equimolar dATP-Mg\(^{2+}\). Aliquots were removed after 1, 3, 10, 20, 60 min from the reaction with Mtr4p (left panel), and after 0.5, 1, 3, 10, 20 min from the reaction with TRAMP (right panel).

(B) Convergence of unwinding and annealing traces of the 16 bp duplex containing 25 nt single-stranded region with Mtr4p (left panel) and TRAMP (right panel). Reactions as shown in panel A (annealing, open circles) and Figure 4.1A (unwinding, filled circles) were quantified and the fraction of single strand was plotted over time. Data were averaged for three independent repeats and error bars indicate the standard deviation. Curves represent best fits to first-order rate law. The unwinding data are identical to those shown in Figure 4.1B. For Mtr4p, \(Amp_{\text{unw}} = 0.846 \pm 0.035\), \(k_{\text{obs, unw}} = 0.06 \pm 0.01\) min\(^{-1}\); \(Amp_{\text{ann}} = 0.047 \pm 0.002\), \(k_{\text{obs, ann}} = 0.13 \pm 0.01\) min\(^{-1}\). For TRAMP, \(Amp_{\text{unw}} = 0.885 \pm 0.026\), \(k_{\text{obs, unw}} = 0.59 \pm 0.06\) min\(^{-1}\); \(Amp_{\text{ann}} = 0.023 \pm 0.003\), \(k_{\text{obs, ann}} = 0.90 \pm 0.38\) min\(^{-1}\).
In the presence of both unwinding and annealing activities, the reaction amplitude was determined by the ratio of the actual unwinding and annealing rate constants (\(Amp_{\text{unw}} = \frac{k_{\text{unw}}}{(k_{\text{unw}} + k_{\text{ann}})}\)) (Yang and Jankowsky, 2005). This ratio varied for TRAMP and Mtr4p and among different duplexes. The lower unwinding amplitude for the 36 bp duplex compared to the 16 bp duplex was due to slower unwinding as well as faster annealing (e.g. for Mtr4p, \(k_{\text{unw}} = 0.052 \pm 0.005 \Rightarrow 0.004 \pm 0.004 \text{ min}^{-1}\), \(k_{\text{ann}} = 0.006 \pm 0.001 \Rightarrow 0.011 \pm 0.003 \text{ min}^{-1}\), Figures 4.1A, B, 4.3, 4.4). All unwinding rate constants shown here are true unwinding rate constants without the annealing component (see Section 10.2. for equations).

In conclusion, Mtr4p harbors annealing activity that becomes dominant in the unwinding reaction as duplex length increases from 16 to 36 bp; when in complex with Trf4p/Air2p, unwinding is more favored over annealing even for the longer duplex.

**Figure 4.5 Unwinding by Mtr4p and TRAMP requires RNA loading strand.**
Unwinding of DNA/RNA hybrid duplexes by Mtr4p (A) and TRAMP (B). The 16 bp RNA duplex containing 25 nt single-stranded region was replaced with DNA on one or both strands. Reactions were performed with 0.5 nM radiolabeled duplex, 400 nM Mtr4p or 100 nM TRAMP and 2 mM dATP-Mg\(^{2+}\). Data were averaged for three independent repeats and error bars indicate the standard deviation. Curves represent best fits to first-order rate law. For Mtr4p with RNA duplex, \(Amp_{\text{unw}} = 0.861 \pm 0.038\), \(k_{\text{obs, unw}} = 0.06 \pm 0.01 \text{ min}^{-1}\); with DNA top strand, \(Amp_{\text{unw}} = 0.878 \pm 0.034\), \(k_{\text{obs, unw}} = 0.07 \pm 0.01 \text{ min}^{-1}\). For TRAMP with RNA duplex, \(Amp_{\text{unw}} = 0.834 \pm 0.041\), \(k_{\text{obs, unw}} = 0.36 \pm 0.05 \text{ min}^{-1}\); with DNA top strand, \(Amp_{\text{unw}} = 0.878 \pm 0.040\), \(k_{\text{obs, unw}} = 0.54 \pm 0.07 \text{ min}^{-1}\).

Having seen that unwinding characteristics of Mtr4p and TRAMP fully conform neither to those of DEAD-box proteins, nor to those of NS3/NPH-II proteins, we next
investigated the requirement for RNA strands in DNA/RNA hybrids. DEAD-box proteins can unwind duplexes containing DNA in either strand but not both (Rogers et al., 2001a; Yang and Jankowsky, 2006) (see Figure 7.1 for data with Ded1p). NS3/NPH-II helicases are unaffected by DNA in the top strand (the strand without 3’ overhang), but inhibited by DNA in the bottom strand (Pang et al., 2002; Kawaoka and Pyle, 2005). For both Mtr4p and TRAMP, the top strand of the 16 bp RNA duplex could be replaced with DNA without impairing unwinding (Figure 4.5). Replacing the bottom strand with DNA, however, abolished unwinding (Figure 4.5). These observations show that unwinding by both Mtr4p and TRAMP requires RNA in the bottom strand.

We noted that the substrate with DNA top strand was unwound slightly faster than the complete RNA duplex. The unwinding by Mtr4p and by TRAMP thus appears to be sensitive to the reduced stability of DNA/RNA hybrids compared to RNA duplexes. Similarly, we have seen that Mtr4p and TRAMP unwound the 36 bp duplex slower than the 16 bp duplex, consistent with decreased activity on more stable duplexes (Figure 4.3).

Current data for Mtr4p and TRAMP illuminate similarities to both translocation-based unwinding represented by NS3/NPH-II helicases and local strand separation seen for DEAD-box proteins. However, we were not able to evaluate processivity or probe whether Mtr4p or TRAMP translocates in the unwinding reaction. Neither Mtr4p nor TRAMP displayed unwinding under single-cycle conditions with excess scavenger RNA (data not shown). Presumably both Mtr4p and TRAMP dissociated from the substrate before a duplex was fully separated or even before unwinding was initiated.

Collectively, our results show that Mtr4p (TRAMP)-mediated unwinding harbors aspects of a polar mechanism (3’->5’), but is sensitive to the length/stability of the duplexes.
The Ski2-like helicase Mtr4p shares features with NS3/NPH-II viral helicases in its requirement for an RNA bottom strand and with DEAD-box proteins in its dependence on duplex length/stability. Trf4p/Air2p stimulates Mtr4p activity without changing these mechanistic characteristics.

4.2.3 Short single-stranded regions promote faster unwinding

To gain further insight into the characteristics of duplex unwinding by Mtr4p and TRAMP, we examined whether and how the length of 3′ single-stranded overhang affected unwinding rate constants. To this end, we used a 16 bp duplex with an overhang of 10 nt instead of 25 nt. At enzyme saturation, Mtr4p unwound the substrate with 10 nt overhang faster than the duplex with 25 nt overhang (Figure 4.6A). TRAMP was similarly affected by the shortened length of the single-stranded region (Figure 4.6B). TRAMP with mutant Trf4p (TRAMP^{Trf4-236p}) also displayed faster unwinding of the duplex with the shorter overhang (Figure 4.7B). In addition, both wtTRAMP and TRAMP^{Trf4-236p} unwound the substrate with 10 nt overhang notably faster than Mtr4p alone (Figures 4.6, 4.7).

![Figure 4.6](image)

**Figure 4.6 Short single-stranded regions (10 nt) promote unwinding by Mtr4p and TRAMP.**

(A) Mtr4p titrations for unwinding of 16 bp duplexes containing 10 nt (gray circles) and 25 nt (black circles) overhangs at the 3′-terminus. Unwinding rate constants were determined from multiple reactions with 0.5 nM radiolabeled RNA, 2 mM dATP-Mg^{2+} and the enzyme concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherm $k_{\text{unw}} = k_{\text{max, E}} [E] / ([E] + K_{1/2, E})$, where [E] represents enzyme concentration. For the duplex with 10 nt single-stranded region, $k_{\text{max, Mtr4p}} = 0.25 \pm 0.03 \text{ min}^{-1}$, $K_{1/2, \text{Mtr4p}} = 499 \pm 106 \text{ nM}$. For the duplex with 25 nt
single-stranded region, $k_{\text{max, Mtr4p}} = 0.090 \pm 0.003 \text{ min}^{-1}$, $K_{1/2, \text{Mtr4p}} = 100 \pm 11 \text{ nM}$, as shown in Figure 4.1C.

(B) TRAMP titrations for unwinding of the 16 bp duplexes containing 10 nt (gray circles) and 25 nt (black circles) single-stranded regions. For the duplex with 10 nt single-stranded region, $k_{\text{max, TR}} = 1.48 \pm 0.24 \text{ min}^{-1}$, $K_{1/2, \text{TR}} = 141 \pm 54 \text{ nM}$. For the duplex with 25 nt single-stranded region, $k_{\text{max, TR}} = 0.84 \pm 0.10 \text{ min}^{-1}$, $K_{1/2, \text{TR}} = 94 \pm 31 \text{ nM}$, as shown in Figure 4.1C.

For Mtr4p, the functional affinity for the duplex with a 10 nt overhang was markedly lower than for the duplex with a 25 nt overhang ($K_{1/2, \text{Mtr4p}} = 100 \pm 11 \text{ nM}$ for 25 nt vs. $K_{1/2, \text{Mtr4p}} = 499 \pm 106 \text{ nM}$ for 10 nt, Figure 4.6A). Affinities of TRAMP for the two substrates differed to a lesser extent ($K_{1/2, \text{TR}} = 94 \pm 31 \text{ nM}$ vs. $K_{1/2, \text{Mtr4p}} = 141 \pm 54 \text{ nM}$, Figure 4.6B).

Thus, Trf4p/Air2p does enhance the affinity of Mtr4p for substrates with relatively short single-stranded regions.

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**Figure 4.7** Duplexes with 10 nt overhangs are unwound faster than duplexes with 25 nt overhangs whether or not adenine sequences are present.

(A) Mtr4p titrations for unwinding of 16 bp duplexes containing 10 nt (gray circles) and 25 nt (black circles) non-A single-stranded regions. Unwinding rate constants were determined from multiple reactions with 0.5 nM duplex, 2 mM equimolar dATP-Mg$^{2+}$ and the Mtr4p concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherm $k_{\text{unw}} = k_{\text{max, E}} [E] / ([E] + K_{1/2, \text{E}})$, where [E] represents enzyme concentration. For the duplex with 10 nt single-stranded region, $k_{\text{max, Mtr4p}} = 0.55 \pm 0.08 \text{ min}^{-1}$, $K_{1/2, \text{Mtr4p}} = 687 \pm 177 \text{ nM}$. For the duplex with 25 nt non-A single-stranded region, $k_{\text{max, Mtr4p}} = 0.15 \pm 0.01 \text{ min}^{-1}$, $K_{1/2, \text{Mtr4p}} = 189 \pm 52 \text{ nM}$.

(B) TRAMP$^{\text{Trf4-236p}}$ titrations for unwinding of the 16 bp duplex containing 10 nt (gray circles) and 25 nt (open circles) A-rich single-stranded regions. Unwinding rate constants were determined from multiple reactions with 0.5 nM duplex, 2 mM equimolar dATP-Mg$^{2+}$ and the TRAMP$^{\text{Trf4-236p}}$ concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherm $k_{\text{unw}} = k_{\text{max, E}} [E] / ([E] + K_{1/2, \text{E}})$, where [E] represents enzyme concentration. For the duplex with 10 nt single-stranded region, $k_{\text{max, TR}} = 1.51 \pm 0.21 \text{ min}^{-1}$, $K_{1/2, \text{TR}} = 126 \pm 39 \text{ nM}$. For the duplex with 25 nt single-stranded region, $k_{\text{max, TR}} = 0.76 \pm 0.06 \text{ min}^{-1}$, $K_{1/2, \text{TR}} = 105 \pm 29 \text{ nM}$.
We next verified that the faster unwinding of duplexes containing a 10 nt single-stranded region was due to length instead of the adenine-rich sequence in the overhangs (repeats of A4 separated by a single nucleotide, see Table 10.1 for sequence). Duplexes containing non-A single-stranded overhangs also showed faster rate constants and lower apparent affinities with 10 nt overhang compared to 25 nt (Figure 4.7A, C). The A-rich sequence does slightly enhance substrate affinity (1.9, 1.6-fold for Mtr4p, TRAMP and 25 nt overhang), while decreasing unwinding rate constants (1.7, 1.5-fold for Mtr4p, TRAMP and 25 nt overhang) (Figure 4.8).

Figure 4.8 Mtr4p and TRAMP slightly discriminate between 25 nt A-rich and non-A single-stranded regions in unwinding.

(A) Mtr4p titrations for unwinding of the 16 bp duplexes containing 25 nt A-rich (filled gray circles) and non-A (open circles) single-stranded regions. The exact sequences are listed in Table 10.1. Unwinding rate constants were determined from multiple reactions with 0.5 nM duplex, 2 mM equimolar dATP-Mg2+ and the Mtr4p concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherm \( k_{\text{unw}} = k_{\text{max},E} [E] / ([E] + K_{1/2,E}) \), where [E] represents enzyme concentration. Data for the duplex with A-rich single-stranded region are the same as shown in Figures 4.1C, 4.6A, where \( k_{\text{max}, \text{Mtr4p}} = 0.090 \pm 0.003 \text{ min}^{-1} \), \( K_{1/2, \text{Mtr4p}} = 100 \pm 11 \text{ nM} \). For the duplex with non-A single-stranded region, \( k_{\text{max}, \text{Mtr4p}} = 0.15 \pm 0.01 \text{ min}^{-1} \), \( K_{1/2, \text{Mtr4p}} = 189 \pm 52 \text{ nM} \), as shown in Figure 4.7A.

(B) TRAMP titrations for unwinding of the 16 bp duplexes containing 25 nt A-rich (filled gray circles) and non-A (open circles) single-stranded regions. Data for the duplex with A-rich single-stranded region are the same as shown in Figures 4.1C, 4.6B where \( k_{\text{max}, \text{TR}} = 0.84 \pm 0.10 \text{ min}^{-1} \), \( K_{1/2, \text{TR}} = 94 \).
± 31 nM. For the duplex with non-A single-stranded region, $k_{max, TR} = 1.30 \pm 0.23 \text{ min}^{-1}$, $K_{1/2, TR} = 150 \pm 53 \text{ nM}$.

Together, our data show that both Mtr4p and TRAMP unwind duplexes with shorter single-stranded regions (10 nt) faster than duplexes with longer single-stranded regions (25 nt). Both Mtr4p and TRAMP have higher affinity for substrates with longer single-stranded regions. Thus, Mtr4p intrinsically distinguishes between the different length of 3’ extensions, and this characteristic is maintained in TRAMP. An increase in unwinding activity with decreasing overhang length is to our knowledge unusual for a helicase.

4.2.4 A minimum of 5 nt single strand is required for duplex unwinding

We and others have previously reported that single-stranded regions 3’ to the duplex are required for unwinding by Mtr4p (Wang et al., 2008; Bernstein et al., 2008), and a duplex with only 1 nt unpaired single strand is not unwound (data not shown). The activity enhancement seen with shortening single strand (10 vs. 25 nt) raised the question what length of the 3’ single strand is necessary for unwinding by Mtr4p and TRAMP. Since 3’ single-stranded extensions could be created by the poly(A) polymerase Trf4p, the question arises whether Trf4p is capable of providing such a landing site for Mtr4p. As the critical overhang length falls between 2-10 nt, how does it relate to the Mtr4p-mediated modulation of Trf4p activity after addition of ~A₄?

To address these questions, we developed a method to simultaneously monitor unwinding and polyadenylation. We used a 16 bp duplex with a single unpaired nucleotide at the 3’ end, which is necessary for efficient polyadenylation (cf. Figures 3.9A, 5.2) but does not support unwinding (data not shown). The top strand contained a biotin moiety at the 3’ end, to enable binding of the duplex to streptavidin beads and at the same time block
polyadenylation on this strand (Figure 4.9A). ATP instead of dATP was used to allow for both polyadenylation and unwinding by TRAMP. As TRAMP polyadenylated the free 3’ end, the single-stranded region was extended. Once the single strand reached the critical length for efficient unwinding, TRAMP was expected to unwind the duplex and release the polyadenylated strand into solution (Figure 4.9A). Consistent with this expectation, we found the supernatant sample enriched with RNAs containing longer poly(A) tails compared to those retained on the beads (Figure 4.9B). Only species with at least 4 adenines were observed in the supernatant, indicating that a minimum of 5 unpaired nucleotides (1 nt overhang + A₄) are required by Mtr4p to start unwinding (Figure 4.9B). Species with > A₄ were still present in the beads sample, as unwinding and polyadenylation likely occurred with similar rate constants and not every substrate molecule with sufficient extension was immediately unwound.
Figure 4.9 Simultaneous polyadenylation and unwinding reaction by TRAMP reveals the minimal oligo(A) length required for duplex unwinding.

(A) Experimental design for simultaneous polyadenylation and unwinding reaction. A 16 bp duplex with a single unpaired nt at the 3'-terminus contains a biotin moiety (represented by the red dot) on the 3' end of the other strand. Asterisk marks the radiolabel. The biotinylated duplex was incubated with streptavidin beads together with 2 mM ATP-Mg$_{2+}$ for 1 hr. Reaction was started by adding TRAMP to a final concentration of 300 nM. 10 min after reaction start, the sample was centrifuged to separate the beads and the supernatant. Numbers in grey circles mark samples analyzed by denaturing PAGE in panel B.

(B) Representative gel (left panel) and quantification (right panel) for the experiment illustrated in panel A. Lane 1: Unreacted duplex. Lane 2: Reacted sample slurry before centrifugation. Lane 3: Beads sample after centrifugation. Lane 4: Supernatant sample after centrifugation. The number of adenines appended (A$_0$…A$_n$) is marked to the left of the gel. Shown in the plot (right panel) are signals for individual adenylated species from Lane 4 divided by the sum of signals from Lanes 3 and 4 for the same species. The sum of Lane 3 and 4 (beads and supernatant samples respectively) faithfully represents the distribution of species in the unsedimented slurry shown in Lane 2 (Figure 4.10). The dashed line indicates A$_4$.

(C) Unwinding reactions for 16 bp duplexes corresponding to A$_3$-A$_5$ (4-6 nt overhang) in panel B. Reactions were performed with 0.5 nM radiolabeled duplex (asterisk: $^{32}$P), 200 nM TRAMP and 2 mM dATP-Mg$_{2+}$ for 10 min. The identical reactions heated at 95°C for 2 min were shown as control for the unwound single strands.

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Figure 4.10 Sample separation after simultaneous polyadenylation and unwinding reaction with TRAMP does not lead to significant loss of material.

Reactions were performed as illustrated in Figure 4.9A. Quantification of the sample slurry before centrifugation (filled circles, Lane 2 in Figure 4.9B), and the sum of beads and supernatant samples after centrifugation (open circles, Lanes 3, 4 in Figure 4.9B) were plotted for each adenylated species (A$_1$…A$_n$). The signal from each species was normalized to the total signal from all species (A$_i$ / Σ (A$_0$…A$_n$)). Data were averaged for three independent repeats and error bars indicate the standard deviation. The substrate (A$_0$, off chart) contained 0.896 ± 0.007 of the total signal in the slurry before centrifugation and 0.892 ± 0.020 of the total signal in the sum after centrifugation.
We next verified that the addition of ~4A prior to unwinding was not an artifact of the reaction being performed on streptavidin beads (Figure 4.9B). Virtually identical results were obtained by simultaneous polyadenylation/unwinding reactions without immobilization (Figure 4.11).

Figure 4.11 Simultaneous polyadenylation and unwinding reaction without beads.
A 16 bp duplex containing a single unpaired nt at the 3’-terminus was radiolabeled on both strands (shown as asterisks). A 2’,3’-dideoxy modification (light gray bar) at the 3’ end of the top strand prevents polyadenylation on that strand. Reactions were performed with 0.5 nM duplex, 150 nM TRAMP and 2 mM equimolar ATP-Mg^{2+}. Two aliquots were simultaneously removed 1, 2, 3, 4, 5, 6, 7, 10 min after the reaction start, and analyzed for polyadenylation and unwinding respectively. (A) Denaturing PAGE showing polyadenylation by TRAMP. Schematics for the species observed are marked to the left. Lines to the right highlight the species plotted in panel C. (B) Semi-denaturing PAGE (see Chapter 10 for protocols) showing unwinding by TRAMP. Schematics for the species observed are marked to the left. (C) Overlay of polyadenylation and unwinding time traces. Fraction of species with at least 1, 3, 4 and 5 adenines were quantified from denaturing gels as illustrated in panel A (open and filled circles) at the times indicated. Fraction of single strand at the same time was quantified from semi-denaturing gels as shown in panel B (squares). Data were averaged for three independent repeats and error bars indicate the standard deviation. The dashed curves represent interpolations of the data points. The unwinding trace surpassed the polyadenylated traces beyond 4 min probably due to difficulty in quantification as more RNA became polyadenylated. It is yet possible that species with less than 3 adenines slowly turned single-stranded and contributed to the trace after 4 min.

Interestingly, the addition of ~4A coincides with the length of oligo(A) where Mtr4p slows polyadenylation and decreases the ATP affinity of Trf4p during polyadenylation (Chapter 3). Our data thus raise the intriguing possibility that Trf4p creates an ~4A (or 5 nt single-strand) “landing site” for Mtr4p, after which Mtr4p unwinds the duplex, facilitated by Trf4p/Air2p. Mtr4p, at the same time, inhibits polyadenylation after 4A whether or not a duplex is present (Chapter 3).

To independently verify the minimum oligo(A) length required for unwinding, we generated a series of 16 bp duplexes with overhangs containing a single nucleotide (uridine) and 3 to 5 adenines (Figure 4.9C). Unwinding of these substrates was measured in reactions with TRAMP without polyadenylation (Figure 4.9C). After 10 min of incubation, less than 5% of the duplex with 3 adenines (4 nt overhang) was unwound by TRAMP, while ~40% of the duplex with 4 adenines (5 nt) and ~70% of the duplex with 5 adenines (6 nt) were unwound. These results confirm that a single-stranded 3’ extension with at least 5 nt (A₅) is necessary for TRAMP to efficiently unwind the duplex. The data also show that the process of polyadenylation is not essential for the dependence on oligo(A) length during unwinding.
Figure 4.12 Mtr4p and TRAMP prefer oligo(A) single-stranded regions in minimum unwinding substrates.

Unwinding of the 16 bp duplexes containing 4-6 nt single-stranded region with (filled circles) or without adenines (open circles) for Mtr4p (A) and TRAMP (B). The exact sequences are listed in Table 10.1. Unwinding rate constants were determined from three independent repeats in the presence of 400 nM Mtr4p or TRAMP, 0.5 nM RNA and 2 mM dATP-Mg^{2+}. Error bars represent the standard deviation.

We next asked whether the adenines played a specific role for unwinding of duplexes with minimal overhangs (4-6 nt) and whether the overhang length and sequence are
recognized by Mtr4p. To this end, we compared unwinding reactions for substrates with and without the adenines for both Mtr4p and TRAMP. Mtr4p displayed a similar dependence on overhang length as TRAMP (~5 nt), indicating that the overhang length was detected by Mtr4p without requiring Trf4p/Air2p (Figure 4.12). At identical enzyme concentrations, both Mtr4p and TRAMP unwound substrates with A faster than substrates with non-A single-stranded regions (Figure 4.12). Oligo(A) sequences in minimal single-stranded extensions apparently provide an advantage for the duplexes in unwinding reactions with Mtr4p and with TRAMP.

To investigate the basis for the higher unwinding activity seen for the substrates with adenine-containing tails, we determined rate constants with increasing concentrations of Mtr4p or TRAMP. We were only able to approach saturation for the substrates with 6 nt of single-stranded region under experimentally attainable enzyme concentrations (Figure 4.13). Nonetheless, the results clearly indicate that the apparently faster unwinding of the A-rich substrate is most likely due to higher affinity for adenine sequences ($K_{1/2, Mtr4p} = 1697 \pm 908$ nM for non-A vs. $K_{1/2, Mtr4p} = 477 \pm 145$ nM for A-rich, $K_{1/2, TR} = 657 \pm 81$ nM for non-A vs. $K_{1/2, TR} = 225 \pm 126$ nM for A-rich), in addition to a small effect on rate constants for TRAMP ($k_{max, Mtr4p} = 0.55 \pm 0.21$ min$^{-1}$ for non-A vs. $k_{max, Mtr4p} = 0.66 \pm 0.10$ min$^{-1}$ for A-rich, $k_{max, TR} = 1.15 \pm 0.10$ min$^{-1}$ for non-A vs. $k_{max, TR} = 2.81 \pm 0.81$ min$^{-1}$ for A-rich). For substrates with either sequence, TRAMP displayed higher affinity than Mtr4p (Figure 4.13). In summary, Mtr4p shows an inherent preference for adenines, which results in higher substrate affinity when the single-stranded region is ~5 nt. This preference for adenines is also seen with TRAMP.
4.3 Discussion

In this study, we have shown that Trf4p/Air2p in the TRAMP complex stimulates the unwinding activity of Mtr4p for a variety of substrates. Short single-stranded extensions (5-10 nt) promote unwinding of RNA duplexes by both Mtr4p and TRAMP. Such single-stranded tails can be created by the polyadenylation activity of Trf4p in TRAMP. The minimum length of single-stranded region coincides with modulation of TRAMP polyadenylation by Mtr4p at ~A4. Adenine sequences are not necessary for unwinding, but do enhance substrate affinity for both Mtr4p and TRAMP.

This report constitutes the first systematic analysis of duplex unwinding by a Ski2-like RNA helicase (Mtr4p) and in a physiological protein complex (TRAMP). Unwinding by Mtr4p has been shown to require single-stranded overhang 3’ to a duplex, consistent with 3’→5’ directional unwinding (Wang et al., 2008; Bernstein et al., 2008). The notion of polar unwinding by Ski2-like proteins is also supported by a crystal structure of the Ski2-like DNA helicase Hel308, where a β–hairpin structure partially opened the first 2 bp of the duplex at the junction between duplex and 3’ single strand (Buttner et al., 2007). In this study, we show that Mtr4p and TRAMP are able to unwind stable RNA duplexes as long as 36 bp and requires an RNA loading strand for unwinding. While these observations are consistent with a translocation-based mechanism represented by NS3/NPH-II proteins, unwinding rate constants decrease with duplex length/stability, reminiscent of DEAD-box proteins which unwind by local strand separation (Section 6.2). Judging from the significant decrease in unwinding rate constant when the duplex length increased from 16 bp to 36 bp, if TRAMP unwinds RNA duplexes through translocation, it will not be nearly as processive as the *Vaccinia virus* NPH-II or the *Hepatitis C virus* NS3 (Jankowsky et al., 2000; Pang et al.,...
It is not known, however, whether interactions with other proteins, such as the nuclear exosome complex, would lead to highly processive unwinding by TRAMP.

We have previously demonstrated that the RNA helicase Mtr4p modulates activities of the poly(A) polymerase Trf4p in the TRAMP complex (Chapter 3). Here, we demonstrate that Trf4p, together with the zinc-knuckle protein Air2p, influences activities of Mtr4p. The unwinding activity of Mtr4p is stimulated in the TRAMP complex for all RNA substrates tested. The affinities of Mtr4p for both RNA and (d)ATP increased in the presence of Trf4p and Air2p. The higher affinity for (d)ATP in TRAMP indicates modulation of the helicase core by Trf4p/Air2p. According to our analysis of polyadenylation activities of TRAMP, Trf4p binds ATP with much higher affinity than Mtr4p does, except for the adenylation steps immediately after the addition of ~4 adenines (Figures 3.6, 3.9). In unwinding reactions for both wtTRAMP and the polymerase mutant TRAMP\textsuperscript{Trf4-236p} (25 nt overhang, Figure 4.1D; 10 nt overhang, data not shown), we consistently observed a Hill coefficient of $n \sim 2$ for (d)ATP. Thus, an ATP-bound state of Trf4p may contribute to an Mtr4p conformation more suitable for ATP binding and duplex unwinding, even though polyadenylation is not necessary for the stimulation. We do not know whether the 3’ end of the RNA substrate was also present at the catalytic center of Trf4p. Interestingly, unwinding by both Mtr4p and TRAMP was faster for duplexes with short single-stranded extensions (5-10 nt) than for long extensions (25 nt), and Trf4p/Air2p enhance substrate affinity only with the short extensions (Figures 4.6, 4.7, 4.13). If Mtr4p binds at the junction between duplex and single-stranded regions as observed in the structure of Hel308 (Buttner et al., 2007), the short extensions could be just long enough to emerge from Mtr4p.
Our results also have implications for the coordination between polyadenylation, unwinding and degradation. Structured substrates with few unpaired residues at the 3’ end are resistant to degradation by the exosome (Liu et al., 2006; Bonneau et al., 2009). Trf4p in the TRAMP complex appends oligo(A) tails to the substrate. Mtr4p facilitates polyadenylation by promoting adenylation and reducing dissociation from the substrate (Figure 3.19). When approximately 4 adenines are present, Mtr4p inhibits the polyadenylation activity of Trf4p, while Trf4p stimulates the unwinding activity of Mtr4p. This concerted action ensures a specific, but minimum oligo(A) tail (~4 nt) on the RNA substrate, which may help the cell to distinguish RNA polyadenylated by TRAMP from mRNAs polyadenylated by Pap1p, as proposed previously (Section 3.3.1). In addition, the oligo(A) tails of ~4 nt may represent a signal for degradation by the exosome (Section 3.3.1).

Duplexes with an unpaired tail of ~30 nt have been reported to be degraded by the exosome in vitro without Mtr4p (Bonneau et al., 2009). A poly(A) tail of this length, however, is likely to be erroneously protected from degradation by the abundant poly(A) binding proteins in vivo. The unwinding activity of Mtr4p can readily provide a ~30 nt unstructured 3’ end in the RNA. Duplexes with long 3’ extensions are unwound slower by TRAMP and show higher affinity compared to duplexes with short extensions. Both effects likely increase the chance for the exosome to degrade the available unstructured region and avoid further accumulation of long single-stranded regions.

We note that all activities of TRAMP and exosome do not necessarily occur within the same TRAMP-exosome assembly. TRAMP appends an average of only ~2 adenines per binding event (Figure 3.19A). The repeated binding/dissociation could allow for adjustments in the modes of binding, with slight preference for oligo(A) sequences during rebinding
(Figures 4.8, 4.13). Crystal structures of Mtr4p revealed a potentially continuous channel for single-stranded RNA from Mtr4p to the exosome core (Jackson et al., 2010; Weir et al., 2010). The 3’ end of the RNA in such a channel would be inaccessible to the polymerase Trf4p, which also acts on the 3’ end. Physical models for the coordination between TRAMP and exosome would require structural information for TRAMP.
Chapter 5

Future directions

Quantitative biochemical analysis of the TRAMP complex

In Chapters 3 and 4, I reported characterization of the polyadenylation and unwinding activities of the TRAMP complex and illustrated crosstalk between the poly(A) polymerase Trf4p and the RNA helicase Mtr4p. This chapter outlines possible future investigations on substrate recognition and ATP utilization by TRAMP and on coordination between TRAMP and the nuclear exosome.

5.1 Recognition of RNA length, structure and sequence by TRAMP

The TRAMP complex targets a wide range of RNAs for processing and degradation in vivo (Section 2.5). It is not clear what defines a TRAMP substrate, but it is reasonable to suspect that the information lies largely in the RNA itself. Indeed, my studies on polyadenylation and unwinding by TRAMP identified effects of RNA length, structure and sequence. The experiments proposed as follows may illustrate how features in an RNA determine recognition and activities by TRAMP.

5.1.1 Minimum length of ssRNA bound by TRAMP

On the 17 nt ssRNA, TRAMP added the 1st adenylate very slowly ($k_1 \sim 0.03 \text{ min}^{-1}$ at 100 nM TRAMP). This apparent slow rate is most likely due to a defect in TRAMP binding (Figures 3.10D, E, 5.1A). The same ssRNA with one less nt at the 3’-terminus (16 nt)
displayed almost no polyadenylation by TRAMP (Figure 5.1B). These results imply that >16 nt ssRNA is minimally required for productive binding by TRAMP. To directly test this hypothesis, equilibrium binding experiments will be performed for TRAMP with different length of ssRNA. RNA binding by Mtr4p alone has been studied with gel shift and fluorescence anisotropy assays, and Mtr4p has been shown to bind 4-5 nt (Bernstein et al., 2008; Bernstein et al., 2010). RNA binding by Trf4p/Air2p can also be measured to see whether the sum of the binding sites for Trf4p/Air2p and for Mtr4p equals the binding site of TRAMP. Alternatively, the binding site of TRAMP and Trf4p/Air2p can be examined by RNA footprinting, which has been used with numerous RNA binding proteins.

![Figure 5.1 Minimum length of ssRNA polyadenylated by TRAMP.](image)

**Figure 5.1 Minimum length of ssRNA polyadenylated by TRAMP.**

(A) wtTRAMP polyadenylation of a 17 nt ssRNA (100 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and 0.5 nM RNA). Same gel as Figure 3.10D.

(B) wtTRAMP polyadenylation of a 16 nt ssRNA (150 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and 0.5 nM RNA).
5.1.2 Polyadenylation of structured RNAs by TRAMP

My data to date indicate that duplex structures constitute better substrates for polyadenylation by TRAMP. As shown above, TRAMP displayed little polyadenylation activity on a 16 nt ssRNA (Figure 5.1B). A 16 bp duplex, however, was efficiently adenylated for the first step (Figure 5.2). Interestingly, the substrate showed significantly reduced adenylation rate constant for the second step, and the peak position was also shifted by 1-2 nt compared to the duplex with 1 nt overhang (Figure 5.2). These results suggest recognition of dsRNA by TRAMP, and a read-out of the presence of the single protruding nucleotide.

![Diagram](image)

Figure 5.2 Structural features of a blunt-ended 16 bp duplex are recognized by TRAMP. (A) wtTRAMP polyadenylation of a blunt-ended 16 bp RNA duplex (150 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and 0.5 nM RNA). The gray bar in the top strand indicates a 3’-terminal 2′,3′-dideoxy residue used to prevent adenylation at this position. (B) Plot show observed adenylation rate constants (\(k_{\text{obs}}\)) for individual adenylation steps with the 16 bp duplex with (open circles) and without 1 nt overhang (filled circles). Reactions performed with 150 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and 0.5 nM RNA. The dashed line indicates the rate constant at A₄.
A 16 bp duplex containing one unpaired nt was also more efficiently polyadenylated than its corresponding single strand (17 nt) (Figures 3.9A, 3.10D, E). Moreover, the 17 nt ssRNA displayed impaired modulation in adenylation rate constants at TRAMP and ATP saturation, and in apparent ATP affinities after A₄ (Figure 5.3). The effect of RNA structure thus persists beyond the initial adenylation until after addition of 4 adenylates.

![Figure 5.3](image)

Modulation of TRAMP activity by Mtr4p is impaired for a 17 nt ssRNA but not for its duplex counterpart.

**A** Adenylation rate constants at TRAMP and ATP saturation ($k_{max}$) for a 17 nt ssRNA (filled circles), compared to the its duplex form (16 bp with 1 nt overhang, open circles). Values were determined from multiple reactions with 0.5 nM RNA and increasing TRAMP and ATP concentrations. Error bars indicate the standard deviation. The gray asterisk represents an estimate for the first $k_{max}$ for the 17 ssRNA, as it did not reach saturation under experimentally attainable TRAMP concentrations. Data for the duplex substrate are the same as in Figure 3.9A. The dashed line indicates the peak rate constant at A₄ for the duplex substrate.

**B** Apparent ATP affinity ($K_{1/2}$) for individual adenylation steps for a 17 nt ssRNA (filled squares), compared to the its duplex form (16 bp with 1 nt overhang, open squares). Data for the duplex substrate are the same as in Figure 3.9A. The dashed line marks A₄.

To better understand TRAMP-mediated polyadenylation of structured vs. unstructured RNAs, detailed kinetic analyses as illustrated in Figures 3.19, 3.20 would be very helpful for attributing differences in polyadenylation reaction to chemical ($k_{i}$) and binding steps ($k_{on}$, $k_{off}$). For the 16 bp and the 23 bp duplexes with a single unpaired nt, I have determined the observed adenylation rate constants, ATP and TRAMP affinities (Figures 3.9, 3.10). An assay for processivity similar to that performed with the 24 nt ssRNA will allow us to calculate the actual adenylation rate constants ($k_{i}$) and the dissociation rate constants ($k_{off}$).
Association rate constants may also be obtained through simulations with the TRAMP titration data \( K_{1/2}^{\text{TRAMP}} \neq \frac{k_{\text{off}}}{k_{\text{on}}}, \) when \( k_{\text{i}} \) is comparable to \( k_{\text{off}} \). Comparing data from the duplexes with data from the corresponding ssRNAs, the impact of duplex structure on adenylation and TRAMP dissociation/association can be dissected for each adenylation step.

Regarding how duplex structures are recognized by TRAMP, a good candidate for dsRNA binding is the KOW domain in Mtr4p. Mtr4p without this domain displayed impaired binding to tRNA\({}_{i}^{\text{Met}}\) (Weir et al., 2010). TRAMP with KOW-deleted Mtr4p can be tested with duplex and single-stranded substrates in polyadenylation reactions. If such a TRAMP complex reacts with the duplex substrates similar to the single-stranded substrates, the KOW domain is key to recognition of structured RNA. The KOW deletion did not abolish binding to Trf4p/Air2p (Weir et al., 2010), yet any mutation/deletion in Mtr4p potentially affects assembly of the TRAMP complex. As long as the KOW-deleted TRAMP displays the modulated adenylation rate constants and ATP affinities on ssRNAs longer than 17 nt (e.g. 24 nt), we can consider the complex to be functional. A role of the KOW domain in dsRNA binding can also be tested by direct RNA-protein binding assays. Air2p may also participate in binding to dsRNA. However, preliminary results showed that Trf4p/Air2p displays little difference in polyadenylation activity for the above-mentioned duplex and single-stranded substrates (data not shown).

5.1.3 Unwinding of structured RNAs by TRAMP

I have illustrated the coordination between polyadenylation and unwinding with the 16 bp-1 nt overhang duplex, and verified the minimum overhang length required for unwinding (Section 4.2.4). It is not clear whether the length of the duplex influences the minimum overhang length observed. Simultaneous polyadenylation/unwinding reactions as
shown in Figure 4.12 can be performed for substrates with different lengths of duplex and single-stranded regions. The results would illuminate questions such as whether the junction between duplex and single-stranded regions is recognized, which then allows measuring of the length of overhang; or whether the same total length of duplex and single-stranded regions is needed regardless of duplex length. I favor the first model because oligo(A) sequence mainly enhances substrate affinity (Figures 4.8, 4.13), duplexes with ~5 nt overhang are expected to be unwound even though the duplexes are shortened. Yet, a certain length of duplex is likely required for proper binding by the TRAMP complex (Section 5.1.2).

Unwinding reactions with different duplex length could also help address whether Mtr4p (TRAMP) translocates on an RNA duplex, and if so in what step size. An answer to this question will help establish further aspects of the unwinding mechanism for Ski2-like helicases, and predict substrates possibly resistant to unwinding by TRAMP. As shown in a pioneer study of the DNA helicase UvrD by Ali and Lohman, if strand separation is rate limiting, the length of the lag phase increases as the duplex becomes longer, and the time courses can be used to determine the kinetic step size of translocation (Ali and Lohman, 1997). If, however, TRAMP separates a duplex without translocation, the unwinding rate constants for different substrates should inversely correlate with duplex stability, similar to the scenario seen for eIF4A (Rogers et al., 2001). Moreover, rate constants and affinities data from unwinding reactions will be compared to data from polyadenylation reactions for a more complete picture of the coordination between the two reactions.
5.1.4 Further delineation of oligo(A) recognition by Mtr4p

Detection of oligo(A) length by Mtr4p is critical for its modulation of TRAMP activity. I have identified E947 in the helical bundle domain to be important for this function (Figure 3.16). E947 makes a single hydrogen bond with N6 of the fourth adenine from the 5’ end (Figure 5.4A). In polyadenylation reactions, TRAMP\textsuperscript{Mtr4(E947A)} weakened interaction with A\textsubscript{4} compared to A\textsubscript{5} (Figure 5.4B), in excellent agreement with the crystal structure (Weir \textit{et al.}, 2010) (Figure 5.4A). The loss of the adenine-specific contact (with N6 of adenine) made the mutant insensitive to the use of GTP instead of ATP (Figure 5.4D). In contrast, wtTRAMP showed a later and broader peak of rate constants with GTP compared to with ATP (Figure 5.4C). For both wtTRAMP and TRAMP\textsuperscript{Mtr4(E947A)}, the slower initial steps with GTP compared to ATP are likely due to adenine-specificity of Trf4p for the incoming ATP as well as for the RNA 3’ end (Figure 2.2).
Figure 5.4 E947 in Mtr4p recognizes adenine.

(A) View of E947 and the 5 nt oligo(A) from molecule B of (Weir et al., 2010), as in Figure 3.16C. The helicase domains are omitted for clarity. Only residues 945-951 and 1026-1030 in the helical bundle domain are shown in gray. E947 makes a single H-bond with N6 of the 4th adenine from the 5’ end.

(B) Substrate affinity for the 4th adenylation step relative to substrate affinity for the 5th step for wtTRAMP (open diamonds) and TRAMP\textsuperscript{Mtr4p(E947A)} (filled diamonds). ss: 24 nt ssRNA, ds: 23 bp duplex with 1 unpaired nt. Apparent substrate affinities were measured from multiple polyadenylation reactions with 0.5 nM RNA, 2 mM ATP-Mg\textsuperscript{2+} and increasing concentrations of TRAMP. Original values were shown for the ssRNA in Figure 3.16F and not shown for the duplex.

(C) Plot shows rate constants for individual adenylation (open circles) and guanylation (filled circles) steps with wtTRAMP. Reactions were performed with 200 nM wtTRAMP, 3 mM ATP-Mg\textsuperscript{2+} (open circles) or GTP-Mg\textsuperscript{2+} (filled circles) and 0.5 mM 24 nt ssRNA. Error bars indicate the standard deviation.

(D) Plot shows rate constants for individual adenylation (open circles) and guanylation (filled circles) steps with TRAMP\textsuperscript{Mtr4p(E947A)}. Reactions were performed with 200 nM TRAMP\textsuperscript{Mtr4p(E947A)}, 3 mM ATP-Mg\textsuperscript{2+} (open circles) or GTP-Mg\textsuperscript{2+} (filled circles) and 0.5 mM 24 nt ssRNA. Error bars indicate the standard deviation.
Curiously, TRAMP<sup>Mtr4(E947A)</sup> still showed preference for adenine/purine sequences when unwinding the 16 bp duplexes with minimum overhangs (Figure 5.5). This activity was consistent with the observation that the E947A mutation does not completely abolish the poly(A) length-dependent modulation of adenylation activity (Figures 3.16, 5.4). According to the crystal structure of Mt<sub>r4p</sub> from Weir et al. (2010), R1026 and R1030 that mediate purine-specific interactions may also play critical roles (Figure 2.3). The first temperature-sensitive mutant <i>mtr4-1</i> (C942Y) also carries a mutation in the helical bundle domain, five residues away from E947. These mutations and other residues around the RNA binding site will be analyzed individually and in combination with E947A. Studies on the mutants will provide an understanding of how Mtr4p recognizes oligo(A) sequence in polyadenylation and unwinding reactions by TRAMP.

From the RNA substrate point of view, oligo(A) sequences enhanced substrate affinity, but not all the adenines necessarily contribute to binding. For unwinding of a 16 bp duplex containing 6 nt overhang, the presence of one or two adenylates may be sufficient for tighter binding to the substrate (Figure 5.6). Effects of adenines in the overhang on
unwinding can be examined with a more comprehensive set of substrates to probe for contributions from individual positions.

![Graph showing unwind rate vs. Mtr4p concentration](image)

**Figure 5.6 Mtr4p recognition of 3’-terminal oligo(A) in unwinding.**

Mtr4p titrations for unwinding of the 16 bp duplexes containing 6 nt single-stranded region with 5 (black circles), 2 (gray circles) or 0 terminal adenines (open circles). The data for substrates with 5 and 0 adenines were the same as in Figure 4.16A. Unwinding rate constants were determined from multiple reactions at 0.5 nM RNA, 2 mM dATP-Mg\(^{2+}\) and the Mtr4p concentrations indicated. Curves represent best fits to the binding isotherm \(k_{\text{unw}} = k_{max, E} \frac{[E]}{[E] + K_{1/2}^E}\), where [E] represents enzyme concentration. For the substrate with A\(_5\), \(k_{max}^{Mtr4p} = 0.66 \pm 0.10 \text{ min}^{-1}\), \(K_{1/2}^{Mtr4p} = 477 \pm 145 \text{ nM}\). For the substrate with A\(_2\), \(k_{max}^{Mtr4p} = 0.35 \pm 0.05 \text{ min}^{-1}\), \(K_{1/2}^{Mtr4p} = 188 \pm 77 \text{ nM}\). With the non-A substrate, \(k_{max}^{Mtr4p} = 0.55 \pm 0.21 \text{ min}^{-1}\), \(K_{1/2}^{Mtr4p} = 1697 \pm 908 \text{ nM}\).

How such substrates behave in polyadenylation will also be investigated. I have demonstrated that a 24 nt ssRNA containing 4 adenylates 5 nt away from the 3’ end did not affect modulation of polyadenylation (Figure 3.15). It is not known whether adenylates within 4 nt of the 3’ end will be counted by Mtr4p even when separated by other sequences, especially guanosines. tRNA\(_{i}^{\text{Met}}\) displayed a peak of adenylation rate constants at A\(_3\) instead of the A\(_4\) seen for the 24 nt ssRNA and the 16 bp duplex with 1 nt overhang (Figures 3.2, 3.4-3.6, 3.9), possibly due to the presence of one A at the 3’ end. Furthermore, preliminary measurements with a 36 nt ssRNA ending with an AG sequence showed a peak of adenylation rate constants at A\(_2\) (data not shown). Such knowledge of the impacts of 3’ sequence on TRAMP activity can readily be implemented in bioinformatics studies of cellular targets in the future.
5.2 ATP utilization in the TRAMP complex

Both the poly(A) polymerase Trf4p and the RNA helicase Mtr4p bind to and turnover ATP. I have established the functional impact of the two enzymes on each other in polyadenylation and unwinding reactions. It is not known how ATP is utilized in these activities of Trf4p and Mtr4p. Experiments proposed in this section aim at illuminating this question.

5.2.1 ATPase activity of Mtr4p and TRAMP

In Chapter 4, I established that Trf4p/Air2p enhances the unwinding activity of Mtr4p in the TRAMP complex. Here, I ask whether Trf4p/Air2p also influenced the ATPase activity of Mtr4p. ATP hydrolysis was measured by performing reactions with \([\gamma^{32}P]ATP\) followed by thin layer chromatography (TLC). I did not detect ATP consumption by Trf4p (to AMP + PP\(_i\)) and only observed ATP hydrolysis by Mtr4p (data not shown). For Mtr4p in the absence of RNA, ATP hydrolysis with an initial velocity \(V_0 = 0.61 \pm 0.17 \mu\text{M/min}\) was seen at 800 nM Mtr4p (Figure 5.7). The activity was not compromised by treatment with RNase (data not shown), indicating that this level of ATPase activity was not caused by inadvertently co-purified RNAs in the Mtr4p preparation. In the TRAMP complex, the ATPase activity in the absence of RNA was elevated by almost one order of magnitude (Figure 4.4), which was also uncompromised by RNase treatment (data not shown). These observations indicate that Trf4p/Air2p in the TRAMP complex markedly enhances the basal rate of ATP hydrolysis by Mtr4p.
I next examined the RNA-stimulated ATPase activity for Mtr4p and TRAMP. The 16 bp duplex containing 25 nt overhang at sub-stoichiometric concentrations (RNA : protein = 1 : 40) stimulated the ATPase activity for Mtr4p alone and to a lesser extent for TRAMP (Figure 5.7). TRAMP showed 4-fold faster ATP hydrolysis than Mtr4p in the presence of RNA. Thus, Trf4p/Air2p is able to enhance both basal and RNA-stimulated ATPase activities of Mtr4p.

![Figure 5.7 Basal and RNA-stimulated ATPase activities of Mtr4p are enhanced in TRAMP.](image)

ATPase assays were performed with 800 nM Mtr4p or TRAMP, 2 mM ATP-Mg²⁺ with or without 20 nM of the 16 bp-25 nt overhang RNA duplex. Initial velocities (V₀) of ATP hydrolysis were determined from reaction time courses.

To illuminate the molecular details of the impact of Trf4p/Air2p on Mtr4p, we further measured the RNA-stimulated ATPase parameters for Mtr4p and TRAMP at a range of concentrations under pre-steady state conditions (enzyme excess over RNA) similar to the unwinding conditions for the same duplex. For Mtr4p, I determined the $k_{cat} = 192 \pm 102$ min⁻¹ (Figure 5.8A), similar to results from Bernstein et al. with single-stranded RNAs (Bernstein et al., 2010). The TRAMP complex showed 4-fold elevated ATPase activity compared to Mtr4p, with a $k_{cat} = 776 \pm 106$ min⁻¹ (Figure 5.8A). This 4-fold enhancement in ATP hydrolysis is less than the 8-fold stimulation in unwinding activity under the same
(d)ATP concentration (2 mM) (Figure 4.1D). As in unwinding, we did not observe a significant difference in affinity between Mtr4p and TRAMP for this duplex with 25 nt unpaired single strand (Figures 4.1C, 5.8A).

![Figure 5.8 Pre-stea...](image)

The apparent ATP affinity of TRAMP in ATPase reactions was close to the apparent dATP affinity measured in unwinding reactions ($K_{1/2}^{dATP} = 0.74 \pm 0.04$ mM for unwinding, Figure 4.1D; $K_{1/2}^{ATP} = 0.51 \pm 0.15$ mM for ATPase, Figure 5.8C). In contrast, the (d)ATP affinity determined for Mtr4p in ATPase reactions was ~5-fold the affinity in unwinding reactions ($K_{1/2}^{ATP} = 1.74 \pm 0.75$ mM for unwinding, Figure 4.2B; $K_{1/2}^{ATP} = 0.36 \pm 0.03$ mM).
for ATPase, Figure 5.8B). These results indicate that ATP hydrolysis and unwinding have similar dependence on (d)ATP concentration for TRAMP but not for Mtr4p.

Figure 5.9 Inhibition of the ATPase activity of TRAMP by excess RNA.
(A) ATP titrations for the ATPase activities of Mtr4p (open circles) and TRAMP (filled circles) in the presence of the 16 bp duplex containing 25 nt overhang. Initial velocities of ATP hydrolysis were determined from multiple reactions at 2 μM RNA, 600 nM Mtr4p or TRAMP and ATP-Mg\(^{2+}\) concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to equation \( V_0 = V_{\text{max}}^{\text{ATP}} [\text{ATP}] / ([\text{ATP}] + K_{1/2}^{\text{ATP}}) \). For Mtr4p, \( V_{\text{max}}^{\text{ATP}} = 40.0 \pm 0.7 \ \mu\text{M min}^{-1}, K_{1/2}^{\text{ATP}} = 3.04 \pm 0.92 \ \mu\text{M} \). For TRAMP, \( V_{\text{max}}^{\text{ATP}} = 5.6 \pm 0.2 \ \mu\text{M min}^{-1}, K_{1/2}^{\text{ATP}} = 0.80 \pm 0.07 \ \mu\text{M} \).
(B) RNA duplex titration for the ATPase activity of Mtr4p. Initial velocities of ATP hydrolysis were determined from multiple reactions at 200 nM Mtr4p, 2 mM ATP-Mg\(^{2+}\) and the RNA concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the binding isotherm \( V_0 = V_{\text{max}}^{\text{RNA}} [\text{RNA}] / ([\text{RNA}] + K_{1/2}^{\text{RNA}}) \). \( V_{\text{max}}^{\text{RNA}} = 5.0 \pm 0.2 \ \mu\text{M min}^{-1}, K_{1/2}^{\text{RNA}} = 0.098 \pm 0.35 \ \mu\text{M} \).
(C) RNA duplex titration for the ATPase activity of TRAMP. Initial velocities of ATP hydrolysis were determined from multiple reactions at 800 nM TRAMP, 2 mM ATP-Mg\(^{2+}\) and the RNA concentrations indicated. Error bars represent the standard deviation. Curves represent best fits to the inhibitor binding equation \( V_0 = V_{\text{max}} - V_I [\text{RNA}] / ([\text{RNA}] + K_I^{\text{RNA}}) \). \( V_{\text{max}} = 9.9 \pm 1.3 \ \mu\text{M min}^{-1}, V_I = 8.7 \pm 1.0 \ \mu\text{M min}^{-1}, K_I^{\text{RNA}} = 1.2 \pm 0.8 \ \mu\text{M} \).

I then investigated how Trf4p/Air2p affected ATP hydrolysis by Mtr4p in multiple turnover conditions (RNA in excess over enzyme). Mtr4p alone displayed higher initial velocity compared to pre-steady state conditions (cf. Figures 5.8A, B and 5.9A, B), due to the higher concentration of enzyme-RNA complexes present. At either ATP or RNA saturation, the \( k_{\text{cat}} \) was lower than the \( k_{\text{cat}} \) with protein excess (\( k_{\text{cat}} = 67 \pm 1 \ \text{min}^{-1} \) or \( 25 \pm 1 \ \text{min}^{-1} \), Figure 5.9A, B vs. \( k_{\text{cat}} = 192 \pm 102 \ \text{min}^{-1} \), Figure 5.8A). The ATP affinity was markedly lower than under pre-steady state conditions (\( K_{1/2}^{\text{ATP}} = 3.0 \pm 0.9 \ \mu\text{M} \), Figure 5.9A vs. \( K_{1/2}^{\text{ATP}} \))
= 0.36 ± 0.03 mM, Figure 5.8B), but is similar to (d)ATP affinity measured in unwinding reactions ($K_{1/2}^{\text{ATP}} = 1.74 ± 0.75$ mM for unwinding, Figure 4.2B). Mtr4p alone may form an RNA-protein-ATP complex irrelevant to actual unwinding when Mtr4p is in excess (e.g. on single-stranded region). Such a complex hydrolyzes ATP at a rate faster than the unwinding complex which has lower ATP affinity and higher RNA affinity. In a not mutually exclusive scenario, both ATP binding and hydrolysis by Mtr4p are inhibited in the presence of excess RNA. An inhibition by RNA was, however, not observed in RNA titrations (Figure 5.9B). These possibilities need to be further investigated with different single strand and duplex substrates.

When I measured ATP hydrolysis by TRAMP under multiple turnover conditions, the reaction was surprisingly slower than with Mtr4p, at a $k_{\text{cat}}$ over 50-fold less than under pre-steady state conditions ($k_{\text{cat}} = 9.3 ± 0.3$ min$^{-1}$) (Figure 5.9A). The slower ATP hydrolysis with TRAMP was apparently due to inhibition by RNA (Figure 5.9C). I did observe a slight stimulation of the ATPase activity of TRAMP by a low concentration of RNA (Figure 5.7), and a monotonous stimulation of the ATPase activity of Mtr4p by RNA (Figure 5.9B). The inhibition of ATPase activity of TRAMP by excess RNA is thus due to the presence of Trf4p and Air2p which likely bind multiple RNA molecules other than what is bound by Mtr4p. While we believe that the pre-steady state results are more relevant in vivo, the multiple turnover results suggest interesting aspects of TRAMP for further study. Still, the apparent ATP affinity was not significantly affected by the reaction condition, and again agreed with apparent dATP affinity measured from unwinding ($K_{1/2}^{\text{ATP}} = 0.80 ± 0.07$ mM, Figure 5.9A vs. $K_{1/2}^{\text{dATP}} = 0.74 ± 0.04$ mM, Figure 4.1D).
5.2.2 Role of ATP hydrolysis in Mtr4p-mediated unwinding

ATP hydrolysis is coupled to translocation in canonical helicases, while ATP hydrolysis is not required for unwinding and is only necessary for enzyme recycling in DEAD-box proteins (Sections 1.4, 6.2). With the prevalence of short RNA duplexes in the cell, the actual unwinding by Mtr4p may often not depend on ATP hydrolysis. Preliminary analyses of Mtr4p with mutations in motif II (DE262,263AA, Mtr4-21p) and motif VI (M504I, Mtr4-20p) displayed ATP-independent unwinding of relatively unstable duplexes such as a 16 bp DNA/RNA hybrid (Figure 5.10). This activity was pronounced especially for Mtr4p\textsuperscript{DE262,263AA} in the absence of ATP (Figure 5.10A), while it was inhibited in the presence of ATP (Figure 5.10B). An RNA-containing species accumulated in the wells even with the presence of EDTA and SDS in the stop buffer (Figure 5.9C, D). This species was quantified as duplex instead of single-stranded in the time courses (Figure 5.9A, B). However, the accumulated species may also represent partially unwound RNA substrates that are not efficiently released by the Mtr4p mutants. Single-stranded RNAs in this case are possibly sequestered from the annealing reaction, allowing a higher unwinding amplitude than later times. The gradual disappearance of the accumulated species correlates with decreased unwinding amplitude, likely due to dominance of annealing over unwinding once the single strands are released into solution. These results suggest that Mtr4p is able to open duplex regions in the absence of ATP, and that ATP-bound and ATP-free Mtr4p favor different modes of RNA binding. Similar results were observed for the *Vaccinia virus* NPH-II (Fairman-Williams, 2009). To further explore the role of ATP hydrolysis in unwinding by Mtr4p, wt and mutants Mtr4p can be studied with ATP analogs that represent different states of ATP hydrolysis. It will be interesting to see whether Mtr4p is able to unwind RNA
duplexes in the presence of a certain ATP analog, and how ATP states influence Mtr4p binding to duplex and single-stranded RNAs.

Figure 5.10 ATP-independent unwinding of a 16 bp DNA/RNA hybrid duplex by Mtr4p mutants. (A) A 16 bp DNA/RNA hybrid duplex containing 25 nt ssRNA overhang was unwound by Mtr4p with mutation in motif II (DE262,263AA, Mtr4-21p, filled diamonds), and in motif VI (M504I, Mtr4-20p, filled squares) in the absence of ATP. Reaction time courses with wtMtr4p in the absence (open circles) and presence of 2 mM ATP-Mg$^{2+}$ (filled circles) were shown for comparison. Reactions were performed with 0.5 nM duplex and 400 nM Mtr4p. Mtr4p was added at reaction start. Curves represent best fits to first-order rate law. (B) ATP-independent unwinding by Mtr4p with mutations in motif II and in motif VI inhibited by ATP. Diamonds: motif II mutant; squares: motif VI mutant. Filled symbols: without ATP; open symbols: 2 mM ATP-Mg$^{2+}$. Data for the reactions without ATP are the same as shown in panel A. Curves represent best fits to first-order rate law. (C) Native PAGE for the ATP-independent unwinding reactions by the motif II and motif VI mutants shown in panel A. Schematics for the duplex and the $^{32}$P-labeled single strand (asterisk: radiolabel) are displayed to the left. A species accumulated in the wells as early as 30 s ($1^{st}$ non-zero time point) after addition of Mtr4p and gradually diminished. This species was conservatively quantified as duplex for the plots in panel A, B. (D) Native PAGE for the ATP-containing reactions with the motif II and motif VI mutants shown in panel B. The species in the wells was conservatively quantified as duplex.
5.2.3 Role of ATP hydrolysis in the modulation of polyadenylation

To investigate whether ATP hydrolysis by Mtr4p is required for the modulation of Trf4p activity, TRAMP with an ATPase-deficient mutant Mtr4p such as Mtr4p^{DE262,263AA} may also prove useful. TRAMP containing mutant Mtr4p will be characterized in detail in polyadenylation reactions as illustrated in Chapter 3. If an activity related to duplex unwinding is responsible for modulation of Trf4p activity, I expect TRAMP^{Mtr4(DE262,263AA)} to show a clear peak of adenylation rate constants at low ATP concentration, but a broader peak at high ATP concentration, as ATP inhibits unwinding-related dynamics in the protein (Figure 5.9B). Mtr4-20p does show diminished ATPase activity when assayed with *E. coli* total RNA (Wang *et al.*, 2008) and results with TRAMP^{Mtr4-20p} are consistent with ATP-inhibited modulation of polyadenylation (Figure 3.13 and data not shown). To corroborate this idea, other mutant TRAMP complexes with ATPase-deficient Mtr4p that are however capable of ATP-independent unwinding similar to the Mtr4p^{DE262,263AA} need to be tested for modulation of polyadenylation under low but not high ATP concentrations. TRAMP mutants unable to catalyze unwinding of unstable duplexes, or able to unwind regardless of ATP concentration can be used as controls.

To study the role of ATP hydrolysis for the modulation of Trf4p activity by Mtr4p, ATP analogs that inhibit Mtr4p but support polyadenylation will also be instrumental. Preliminary experiments with ADPNP and ADP-BeF₃, however, did not show polyadenylation with either TRAMP or Trf4p/Air2p (Raul Jobava, unpublished results). Other commonly used ATP analogs such as ADPCP, ATP-γ-S remain to be tested.
5.2.4 ATP status of Trf4p during stimulation of Mtr4p-mediated unwinding

(d)ATP titrations for wtTRAMP and TRAMP^{Trf4-236p} in unwinding showed a hill coefficient of ~2, suggesting that Trf4p is also bound by (d)ATP during unwinding, consistent with the higher (d)ATP affinity of Trf4p compared to Mtr4p (Section 4.3). To directly examine whether ATP-bound Trf4p is involved in TRAMP-mediated unwinding, non-reactive ATP analogs (e.g. ADPNP) with fluorescent labels may be tested for binding to Trf4p (and Trf4-236p) and Mtr4p in the TRAMP complex. ATP analog binding to Trf4p is expected to precede ATP analog binding to Mtr4p. It may then be possible to engineer Trf4p mutants that weaken ATP binding and assess whether ATP-binding by Trf4p is necessary for the stimulation of the unwinding activity of Mtr4p in TRAMP.

The similar unwinding activities of wtTRAMP and TRAMP^{Trf4-236p} with dATP argue that the polymerase activity of Trf4p is dispensable for its stimulation of unwinding activity. It is not clear whether a reaction intermediate during ATP hydrolysis by Trf4p (ATP -> AMP + PPi) confers additional enhancement in the activity of Mtr4p. To answer this question, simultaneous polyadenylation/unwinding reactions in the presence of ATP will be mathematically modeled to determine unwinding rate constants with individual length of single-stranded overhang, and compared to reactions without polyadenylation (with dATP). Measured values for polyadenylation rate constants will be incorporated into the model to reduce the number of parameters. Moreover, 3’ dATP may be used alone or in combination with ATP to terminate polyadenylation without inhibiting unwinding. The data will allow additional calibration of the kinetic model for more accurate determination of unwinding rate constants in the context of polyadenylation.
5.3 Coordination between TRAMP and exosome

5.3.1 Effect of TRAMP on degradation by the nuclear exosome

Studies with TAP-purified TRAMP and exosome from *S. cerevisiae* indicate that TRAMP stimulates degradation of RNAs by the exosome (LaCava *et al.*, 2005; Vanacova *et al.*, 2005). It is not known whether the apparent stimulation is due to the presence of TRAMP, TRAMP-catalyzed polyadenylation, unwinding activities, or all of the above. It is also not clear how TRAMP affects individual activities of the exosome, i.e. the endonuclease and exonuclease activities of Rrp44p, the exonuclease activities of Rrp6p, and whether such effects are exerted on isolated components or only in the context of the exosome core. With our recombinant TRAMP complex, and reconstituted and purified exosome from the laboratory of Dr. Christopher D. Lima (Liu *et al.*, 2006), we are going to explore these questions.

Purified Rrp44p and Rrp6p alone or together with the 9-component exosome core will be first assayed with ssRNA model substrates. Rate constants for individual steps of degradation can be determined with a method similar to the analyses of polyadenylation by TRAMP (Appendix 2), or with a mathematical model developed by Hartung *et al.* (2010) for the archaeal exosome. Endonuclease- and exonuclease-inactive mutants of Rrp44p and Rrp6p will serve as controls for non-specific degradation (Dziembowski *et al.*, 2007; Lebreton *et al.*, 2008; Schneider *et al.*, 2009; Schaeffer *et al.*, 2009; Midtgaard *et al.*, 2006). Possible effects of RNA structure and sequence on degradation will also be examined. If certain substrates can only be degraded by one exosome activity and not by others, it will greatly simplify our kinetic analysis of the exosome (Section 5.3.2). The basic characterization of Rrp44p and Rrp6p with and without the 9-component exosome core at
single-nucleotide resolution will serve as a starting point for understanding of the impact of TRAMP on exosome activity.

TRAMP will be added without nucleotide, with dATP or ATP, to investigate effects of TRAMP proteins, unwinding activity and polyadenylation/unwinding activities, respectively, on exosome-mediated degradation. A qualitative study with TAP-purified TRAMP and recombinant Rrp6p by Callahan and Butler reported that TRAMP stimulates Rrp6p activity independent of ATP or polyadenylation (Callahan and Butler, 2010). Such low-resolution information will be our goal in the initial analyses of TRAMP and exosome. Kinetic measurements of TRAMP and exosome activities at single nucleotide resolution will be described in Section 5.3.2. Reaction conditions may need to be optimized for robust activities of both exosome and TRAMP. It will also be important to determine the affinity of TRAMP for the exosome core, Rrp44p and Rrp6p. TRAMP is likely to influence degradation both as an independent entity and in complex with exosome components. These two possibilities can be distinguished by varying the concentration ratio of TRAMP and exosome components.

5.3.2 Kinetic dissection of simultaneous polyadenylation/degradation

To quantitatively understand the coupling between TRAMP-mediated polyadenylation and exosome-mediated degradation, we are going to analyze individual steps of the polyadenylation/degradation reaction. The reaction can be described with a simple kinetic scheme (Figure 5.11). In this scheme, both polyadenylation and degradation are considered to be chemically irreversible; forward steps represent polyadenylation, reverse steps represent degradation. A mathematical model for a similar reaction scheme (RNA length from 4 to 30 nt) has been reported by Hartung et al., although the authors were
studying the archaeal exosome only and a model without the forward steps sufficiently described their data (Hartung et al., 2010). This model can probably be used for our analysis of simultaneous reactions with TRAMP and the exosome with minor adaptations. If necessary, I do not expect major difficulties in developing our own mathematical solution for the reaction scheme, either. This model only distinguishes RNAs by length, allowing the reaction time course to be visualized by denaturing PAGE with 5’ end-labeled RNA, as shown for TRAMP alone (Chapter 3). I have shown, however, that polyadenylation by TRAMP is modulated by the number of 3’ adenylates. To approximate polyadenylation of RNAs of the same length but different number of 3’ adenylates with a single adenylation rate constants may not adequately describe the reaction, and the observed rate constants are composite rate constants that may be hard to interpret (Figure 5.10). A similar concern exists for degradation. Nonetheless, this simple method can be useful in comparing highly similar substrates.

![Polyadenylation by TRAMP](image)

**Figure 5.11 Kinetic scheme for simultaneous polyadenylation and degradation reaction considering RNA length only.**

R<sub>1...n</sub> represent ssRNA of 1-n nt. n is larger than and unrelated to the original length of the substrate. Forward rate constants k<sub>1...n</sub>-1 describe polyadenylation by TRAMP, reverse rate constants k<sub>n-1</sub> describe degradation by exosome.

To incorporate potential sequence effects on polyadenylation/degradation into our model, I plan to study the reaction with the deep sequencing technique. The RNA substrate will give rise to multiple intermediates in the presence of TRAMP and exosome, with varying number of 3’ adenylates (Figure 5.11A). At least initially, adenosine sequences in the
RNA substrate should be avoided, because they would be indistinguishable from oligo(A) appended by TRAMP (Figure 5.11A). The reaction scheme includes all the species containing varying length of the original sequence plus an oligo(A) tail (Figure 5.11B). For deep sequencing, TRAMP and exosome will be incubated with the ssRNA for different durations of time in the presence of ATP, the reactions will be quenched and RNAs will be ligated to DNA adapters (Wang et al., 2009; Marguerat and Bahler, 2010). The DNA adapters contain barcodes for each reaction, and different reactions can thus be sequenced together. The data require a more sophisticated mathematical model than the one used for the reaction scheme considering length only (Figures 5.10, 5.11B). The use of the deep sequencing technique, however, will provide a sufficient amount of data for accurate determination of the dozens of rate constants.

**Figure 5.12 Simultaneous polyadenylation and degradation reaction considering RNA length and sequence.**

(A) Intermediates for polyadenylation/degradation of a model substrate (24 nt), shown for every 4 nt of the original sequence degraded. The length of the original substrate remaining is listed on the right. Wherever there is an A in the substrate, there is ambiguity regarding whether it is from the substrate or appended by TRAMP.
(B) $R_{1...m}$ represent 1-m nt long 5' remnants of the substrate $R_m$. $A_{1...n}$ indicate oligo(A) tails of 1...n nt. Rate constants $k_{ij}$ and $k_{ji}$ ($i = 1...m, j = 1...n$) describe addition and degradation of oligo(A) respectively. Rate constants $k_{ii}$ ($i = 1...m$) represent effective degradation of the substrate.
Chapter 6

Introduction

The DEAD-box helicase Ded1p

6.1 Conservation and functions of the S. cerevisiae DEAD-box protein Ded1p

DED1 (definition of essential domain 1) was isolated as an essential gene in S. cerevisiae by Struhl (1985). Ded1p contains all the conserved motifs of DEAD-box proteins (Section 1.3.1). In S. cerevisiae, a highly similar but nonessential DEAD-box protein, Dbp1p (72% identical), rescues ded1Δ when overexpressed (Jamieson and Beggs, 1991). Ded1p homologs include Belle in Drosophila melanogaster (Johnstone et al., 2005), An3 in Xenopus laevis (Askjaer et al., 2000), PL10 in Mus musculus (Leroy et al., 1989), and DDX3X, DDX3Y in Homo sapiens (Abdelhaleem et al., 2003; Sekiguchi et al., 2005). Ded1p is also related to Dbp2p in S. cerevisiae, Vasa in D. melanogaster, DDX4 and DDX5 (p68) in humans, etc. (Linder, 2003; Kurimoto et al., 2005).

Ded1p has been implicated in a number of cellular processes such as pre-mRNA splicing (Jamieson and Beggs, 1991; Stevens et al., 2002), mRNA export, translation (Chuang et al., 1997; de la Cruz, 1997) and ribosome biogenesis (Thuillier et al., 1995; Schafer et al., 2003). DED1 was isolated as a suppressor for a mutation in the U5 snRNP protein Prp8p (prp8-1) (Jamieson and Beggs, 1991). Consistently, Ded1p has been found in the penta-snRNP, and mutations in Ded1p caused splicing defects (Stevens et al., 2002).

Evidence for Ded1p’s involvement in mRNA export came mainly from its homologs. The Xenopus homolog of Ded1p, An3, shuttles between the nucleus and the cytoplasm and...
interacts with the export receptor CRM1 (Askjaer et al., 1999). An3 lacking the putative nuclear export signal (NES) (An3Δ21) or containing a mutation in the helicase motif II (E389Q) accumulated in the nucleus (Askjaer et al., 1999; Askjaer et al., 2000). The human homolog, DDX3 also shuttles between the nucleus and the cytoplasm, binds CRM1 and localizes to nuclear pores (Yedavalli et al., 2004). DDX3 is required for Rev/RRE-dependent export of HIV-1 RNA (Yedavalli et al., 2004). Yeast Ded1p itself does have the conserved (NES) (Heath Bowers, unpublished observation), and has been shown to interact with Npl3p (Hurt et al., 2004; Tardiff et al., 2007), a protein involved in multiple processes including mRNA export (Lee et al., 1996; Windgassen et al., 2004; Dermody et al., 2008; Kress et al., 2008).

Regarding Ded1p’s role in translation, genetic interactions have been reported between DED1 and genes encoding translation initiation factors (eIF4A, eIF4B, eIF4E and eIF4G) (de la Cruz et al., 1997), repressor of translation initiation (p20) (de la Cruz et al., 1997), and mRNA decay factors (DCP2, DHH1 and KEM1) (Beckham et al., 2008). Ded1p’s involvement in translation was further supported by large-scale affinity purification results (Krogan et al., 2004; Gavin et al., 2006; Collins et al., 2007), and by diminution of S35-incorporation and polysomes in temperature-sensitive mutants (Chuang et al., 1997; de la Cruz et al., 1997). Moreover, Ded1p has been reported to localize to P-bodies (processing body), and overexpression of Ded1p increased P-body formation and inhibited growth (Beckham et al., 2008).

Ded1p was present in pre-ribosomal particles, suggesting a function of Ded1p in ribosome biogenesis (Stevens et al., 2002). This was supported by dosage compensation of a truncated subunit of RNA polymerase III (Pol III) by Ded1p (Thuillier et al., 1995), although
Pol III is responsible for transcription of RNAs such as tRNAs and U6 snRNA in addition to the ribosomal 5S rRNA (Dieci et al., 2007).

6.2 Unwinding mechanism of Ded1p

6.2.1 Biochemical activities of Ded1p

Ded1p has been shown to have ATP-dependent RNA duplex unwinding (Iost et al., 1999; Yang and Jankowsky, 2005), protein displacement (Fairman et al., 2004; Bowers et al., 2006) and ATP-independent strand annealing activities (Yang and Jankowsky, 2005). Ded1p is able to promote RNA structural conversion with its unwinding and annealing activities (Yang et al., 2007b), and can function as an RNA chaperone for group I, group II intron splicing (Halls et al., 2007; Del campo et al., 2009). The laboratory of Dr. Eckhard Jankowsky focuses on elucidating the mechanism of unwinding by Ded1p, the insights from which may be later applied to our understanding of other activities, and to other DEAD-box proteins. The unwinding activities of several DEAD-box proteins, SrmB, CYT-19, Ded1p and Mss116p have been shown to correlate with their RNA chaperone activities (Del Campo et al., 2009).

6.2.2 Canonical translocation-based unwinding

As discussed in Section 1.4, all SF2 RNA helicase families other than DEAD-box proteins at least prefer to have a single-stranded region (overhang) at the 3’ terminus of a duplex. This requirement for a specific overhang orientation is consistent with a unidirectional translocation mechanism of unwinding established for DNA helicases such as RecBCD, UvrD (Dillingham and Kowalczykowski, 2008; Singleton et al., 2007), and
directly shown for NS3/NPH-II family of SF2 RNA helicases (Dumont et al., 2006; Myong et al., 2007). In this “canonical” mechanism, the strand containing the single-stranded overhang is both the loading strand for initial binding, and the tracking strand for translocation (Figure 6.1). The strict requirement for the overhang at a defined orientation relative to the duplex (3’ terminal) reflects the built-in direction of translocation (3’->5’). Translocation requires an intact sugar-phosphate backbone in the loading strand, but is not affected by nicks in the non-loading strand (Pyle, 2008). ATP is consumed during initiation of unwinding from the overhang (Fairman-Williams, 2009), while ATP turnover is tightly coupled to translocation during duplex unwinding (Pyle, 2008).

![Figure 6.1 Translocation-based duplex unwinding](image)

**Figure 6.1 Translocation-based duplex unwinding.**
Lines represent RNA strands, the oval marks the helicase and the black rectangle indicates the ATP. Only a monomeric enzyme is displayed, but canonical helicases have also been shown to function as oligomers (Lohman et al., 2008). The helicase binds to the single stranded region and in multiple, ATP-dependent consecutive steps translocates towards the opposite end. In the process, the complementary strand is removed. Each translocation step consists of multiple processes including ATP binding and hydrolysis, a power stroke to produce the forward movement, and dissociation of the products of the ATP hydrolysis. Modified with permission from Elsevier (Jankowsky, 2011).
6.2.3 Single-stranded overhangs facilitate loading without translocation

DEAD-box proteins, however, appear to be different. Most of the DEAD-box proteins studied so far unwind duplexes with an overhang at either 3’ or 5’ terminus (e.g. Rogers et al., 2001a; Huang and Liu, 2002; Bizebard et al., 2004). These observations raise the question whether unwinding by DEAD-box proteins is achieved by a mechanism other than unidirectional translocation.

Fellow graduate students have made tremendous progress in deciphering the mechanism of unwinding by the DEAD-box protein Ded1p. A study by Quansheng Yang excluded the possibility that Ded1p unwinds by translocation from single-stranded overhang to the duplex region (Yang and Jankowsky, 2006). Either but not both strands of the RNA duplex could be replaced by DNA without impairing unwinding, whether the overhang is at the 3’ or 5’ terminus. If Ded1p unwound by translocation, unwinding of duplexes with a DNA loading strand would have predicted translocation on DNA, inconsistent with the inability of Ded1p to unwind DNA duplexes. This mechanism was made even more unlikely by the use of a DNA loading strand with a flipped overhang. A helicase translocating 3’->5’ on the DNA overhang would have been hindered by the 5’->3’ portion in the duplex. Instead, Ded1p unwound this duplex as efficiently as the corresponding duplex without the flip. Furthermore, a multi-piece duplex formed between a DNA loading strand and a DNA non-loading strand followed by an RNA non-loading strand showed no unwinding of the DNA portion proximal to the overhang, but was unwound at the distal RNA/DNA portion. In all these cases, the presence of a single-stranded overhang, whether RNA or DNA, was required for efficient unwinding of the duplex region. Given that DNA can be bound by Ded1p, the data suggested that single-stranded overhangs function to recruit Ded1p to duplex regions.
This idea was corroborated by an experiment where no covalent linkage between ssRNA and dsRNA was used. The ssRNA and dsRNA both had biotin at one end and were held in proximity to one another through immobilization on streptavidin protein. The co-presence of the ssRNA on streptavidin, but not in solution facilitated unwinding of the duplex. Thus, single-stranded RNA (or DNA) only needs to be in proximity to an RNA duplex to promote unwinding by Ded1p; no continuous ribo-phosphate backbone is required.

6.2.4 Localized initiation of unwinding by Ded1p and Mss116p within duplex regions

Now that unwinding by Ded1p does not involve translocation, does it still start from one end of the duplex? Another study by Quansheng Yang demonstrated that unwinding by Ded1p and another DEAD-box protein Mss116p can be initiated either from the ends or from internal regions of a duplex (Yang et al., 2007a). A series of chimerical DNA/RNA duplexes harboring short RNA regions in one strand were tested for unwinding by Ded1p. The RNA patch was placed at the ends and in the middle of an otherwise DNA duplex. Ded1p efficiently unwound all substrates, regardless of the orientation of the single-stranded overhang, while the NS3/NPH-II family protein NPH-II did not. The presence of as few as 2 nt of RNA in the 16 bp DNA duplex lead to unwinding. These data indicate that unwinding by Ded1p can be initiated from both terminal and internal regions of a duplex. Similar results were obtained for the DEAD-box protein Mss116p (Yang et al., 2007a), an RNA chaperone that facilitates splicing and translation in the yeast mitochondria (Huang et al., 2005; Halls et al., 2007). The mode of unwinding represented by Ded1p and Mss116p was termed local strand separation (Yang et al., 2007a) (Figure 6.2). In this model, the helicase opens a few base pairs in the duplex, and the remaining base pairs dissociate spontaneously (supported by slower unwinding of more stable duplexes, Rogers et al., 1999; Yang and Jankowsky, 2005;
Yang and Jankowsky, 2006; Chen et al., 2008 and Quansheng Yang, unpublished observations). Unwinding rate constants significantly decreased when the length of the RNA region was reduced from 10 nt to 5 nt and then to 2 nt. Notably, overhangs no longer enhanced unwinding when the RNA patch was reduced from 10 nt to 5 nt. It was thus suggested that the 10 nt RNA region enabled unwinding by multiple Ded1p molecules loaded by the overhang, while the 5 nt RNA region could only accommodate a monomer Ded1p.

![Figure 6.2 Local strand separation by DEAD-box proteins.](image)

Step 1: the DEAD-box protein binds to the duplex either at a terminal or an internal region. This loading is facilitated by single-stranded regions if the RNA segment in the duplex is not shorter than a critical length between 10 and 5 nucleotides and the single-stranded region also exceeds a critical length between 5 and 15 nt. Step 2: the duplex is opened locally in an ATP-dependent reaction. This opening can occur anywhere in the duplex, provided RNA residues are accessible. The number of base pairs opened is hypothetical. Step 3: dissociation of remaining base pairs. For longer and/or more stable duplexes this step will greatly affect the overall efficiency of the strand separation. The overall rate of duplex unwinding will thus depend on loading efficiency (step 1, accounting for effects of single stranded regions and effects of enzyme concentrations), the rate by which the duplex is locally opened (step 2, accounting for effects of ATP concentration), and the lifetime of this locally opened state, as well as on the stability of remaining base pairs (step 3, accounting for the effect of duplex length and/or stability). Adapted with permission from Elsevier (Yang et al., 2007a).
6.2.4 ATP hydrolysis is required not for unwinding, but for enzyme recycling

In canonical helicases, the ATPase cycle is coupled to translocation (Figure 6.1). It was not known how ATP is utilized by DEAD-box proteins such as Ded1p, which unwinds by local strand separation. Another fellow graduate student, Fei Liu, found that ssRNA regions stimulate the ATPase activity of Ded1p significantly more than dsRNA regions, without correlation with the rate of unwinding (Section 7.1). Moreover, ATP binding, but not ATP hydrolysis is required for duplex unwinding by Ded1p and other DEAD-box proteins tested; ATP hydrolysis is, however, necessary for enzyme recycling (Liu et al., 2008). The ground state ATP analog ADP-BeF₅ was sufficient for unwinding, while the non-hydrolyzable analog ADPNP and the transition state analog ADP-αF₄ were not. These data suggested that a state of ATP before hydrolysis is responsible for unwinding and that ATP hydrolysis is likely a consequence of opened single-stranded regions which stimulate catalysis. All three analogs were able to form a stable complex with Ded1p on the RNA substrate. For ADP-BeF₅, which does enable pre-steady state duplex unwinding, such a stable complex was observed even after unwinding, suggesting a defect in recycling of Ded1p. As a consequence, ADP-BeF₅ did not support Ded1p-mediated unwinding under multiple turnover conditions. ATP, on the other hand, can be hydrolyzed and thereby enables rapid dissociation of Ded1p from the RNA.
6.3 Similar mechanisms in other DEAD-box proteins

6.3.1 Facilitated loading to duplex regions

The translation initiation factor eIF4A is able to unwind blunt-ended duplexes, but unwinding is facilitated by the presence of single-stranded overhangs (Rogers et al., 2001b). The length of overhang necessary for maximum unwinding amplitude (after 15 min) increased from ~8 nt for eIF4A alone, to 10, 25 and 16 nt respectively in the presence of eIF4H, eIF4B and within eIF4F (Rogers et al., 2001b). It is not known whether eIF4H, eIF4B, and eIF4G (in eIF4F) facilitated loading of eIF4A to the duplex region through their binding to the overhang, or they increased unwinding through other mechanisms such as preferential binding to ssRNA. The mitochondria protein Mss116p is able to directly bind to blunt-ended duplexes (Mohr et al., 2008), but unwinding is saturated at lower enzyme concentrations in the presence of single-stranded overhang (Mohr et al., 2008; Yang et al., 2007a), consistent with a role of the overhang in facilitating enzyme loading.

For the N. crassa homolog of Mss116p, CYT-19, unwinding was stimulated by a nearby duplex as well as by its single-stranded form (Tijerina et al., 2006). The arginine-rich C-terminus of CYT-19 was responsible for the stimulation by adjacent RNA (Grohman et al., 2007). Note that the C-terminus of Ded1p is also rich in arginines, but it is not clear whether the C-terminus functions in RNA binding or protein oligomerization or some other processes.

6.3.2 Local strand separation

Based on a crystal structure of the D. melanogaster DEAD-box protein Vasa, Sengoku et al. proposed an RNA-bending mechanism for unwinding (Sengoku et al., 2006). The 7 nt ssRNA observed in the structure was sharply bent between the 5th and the 6th
nucleotide from the 5’ end, due to a steric clash with α7 helix harboring motif Ic in the N-terminal RecA-like domain. The bent ssRNA would be incompatible with duplex formation and suggested immediate remodeling of RNA structure upon binding of Vasa in the presence of ATP. Notably, the non-hydrolyzable analog ADPNP was used in the structure, which did not support unwinding in Fei Liu’s study on Ded1p, Mss116p and eIF4A.

Crystal structures of Mss116p revealed a second wedge that kinks the RNA as well (Del Campo and Lambowitz, 2009) (Figure 6.3). 10 nt of ssRNA (poly(U)) was bound by Mss116p. The α-helix containing motif Ic bent between the 6th and the 7th nucleotides, as in structures of Vasa (Sengoku et al., 2006), eIF4AIII (Bono et al., 2006; Andersen et al., 2006), etc. An Mss116p-specific α-helical C-terminal extension bent the RNA between the 3rd and the 4th nucleotides. The structures further led to the belief that DEAD-box proteins
unwind by locally disrupting RNA duplexes, which was demonstrated for Ded1p and Mss116p (Yang et al., 2007a). It was puzzling, however, that all the ATP analogs used, ADPNP, ADP-BeF₃ and ADP-AlF₄ displayed superimposable conformations of Mss116p (Del Campo and Lambowitz, 2009), despite the previous report that only ADP-BeF₃ allows unwinding (Liu et al., 2008). Yet, the structures are consistent with the observation that all the analogs were able to induce formation of a stable complex of the enzyme on ssRNA (Liu et al., 2008; Liu, 2010).

6.3.3 Unwinding without obligatory ATP hydrolysis

In addition to Ded1p, Fei Liu demonstrated that two other DEAD-box proteins from S. cerevisiae, Mss116p and eIF4A, were able to unwind duplexes in the presence of ADP-BeF₃ (Liu et al., 2008).

A study from the laboratory of Dr. Rick Russel on CYT-19 analyzed the relationship between ATP utilization and unwinding (Chen et al., 2008). For a 6 bp duplex under low magnesium conditions, less than 1 ATP molecule was hydrolyzed per strand separation event. These data indicate that ATP hydrolysis is not obligatory for duplex unwinding, corroborating Fei Liu’s finding that only ATP binding is necessary (Liu et al., 2008).

6.4 Further questions on Ded1p addressed in this thesis

6.4.1 Same or different Ded1p unit(s) in loading and unwinding

As pointed out by Mayas and Staley (2006), a couple of questions followed Quansheng Yang’s elucidation of the role of single-stranded overhangs in loading Ded1p to duplex regions (Yang et al., 2006). It was not clear whether the same unit of Ded1p bound to
the overhang and then transferred to the duplex, or a different unit was loaded onto the
duplex through oligomerization of Ded1p. It was also not known whether Ded1p specifically
requires ssRNA for loading or can exploit a protein instead. Chapter 7 of my thesis
demonstrated the existence of distinct Ded1p units for loading and unwinding, and showed
that pre-bound Ded1p-ADPNP facilitated unwinding by additional Ded1p units.

6.4.2 Binding site size of Ded1p on RNA in the presence of ATP analogs for different
states of ATP hydrolysis

The structure of Vasa showed contacts to 5 nt ssRNA (Sengoku et al., 2006). Similar
results were observed for eIF4AIII in EJC (6 nt, Bono et al., 2006; Andersen et al., 2006) and
DDX19 (6 nt, Collins et al., 2009). Mss116p, however, binds a total of 10 nt ssRNA. Given
that Ded1p and Mss116p behaved similarly when the length of RNA in a DNA/RNA chimera
duplexes was reduced from 10 nt to 5 and 2 nt (Yang et al., 2007a), they likely share a
similar binding site size. Fei Liu’s study indicated that only the ground state analog ADP-
BeF$_x$ mimics an unwinding-competent state of ATP (Liu et al., 2008), but little difference
was observed in the crystal structures of Mss116p bound to ssRNA and the non-hydrolyzable
analog ADPNP, the ground state analog ADP-BeF$_x$ or the transition state analog ADP-AlF$_4$.
Chapter 8 of this thesis showed that the binding site size of Ded1p in complex with these
analogs on ssRNA are all ~10 nt. Ded1p-ADP-BeF$_x$ and Ded1p-ADP-AlF$_4$ were able to bind
5 nt ssRNA, likely in a not fully closed conformation that allows dissociation of the analog.
ADP-BeF$_x$ and ADP-AlF$_4$ led to the binding of more Ded1p molecules on a given RNA than
ADPNP, possibly through formation of Ded1p oligomers.
Chapter 7

Unwinding by Ded1p involves distinct loading and unwinding enzyme units

7.1 Introduction

As discussed in Chapter 6, this chapter addresses the question whether the same unit of Ded1p is bound to single-stranded regions and then transferred to duplex regions, or another unit of Ded1p is loaded directed onto the duplex regions through oligomerization of Ded1p.

Single-stranded RNAs stimulate the ATPase activity of Ded1p to a greater extent than double-stranded RNAs (Liu, 2010). However, the rate of ATP hydrolysis on single-stranded overhangs did not correlate with how fast the duplexes were unwound (Liu, 2010) (Figure 7.1). While DNA did not stimulate ATP hydrolysis, DNA/RNA hybrid duplexes containing DNA overhangs were unwound as fast as those containing RNA overhangs. The ATP turnover stimulated by ssRNAs was largely unnecessary for unwinding. To us, this observation is a hint that Ded1p units bound to the overhang may not be the same unit that performs the actual unwinding.

To test this hypothesis, we sought to differentially inhibit Ded1p on single strand and duplex portions with the non-hydrolyzable ATP analog ADPNP. ADPNP not only inhibits ATP hydrolysis, but also locks Ded1p in a stable non-productive complex on single-stranded RNA ($k_{off} \sim 10^{-3} \text{ min}^{-1}$ for both Ded1p and ADPNP, Fei Liu, unpublished observation). In gel shift assays, binding of Ded1p-ADPNP to a duplex with single-stranded overhang was not
appreciably different from binding to the single strand alone (Figure 8.3), suggesting unstable interaction between Ded1p-ADPNP and the duplex region.

![Diagram](image)

**Figure 7.1** ATP hydrolysis on ssRNA does not correlate with duplex unwinding (Liu, 2010). Kinetics parameters of ATP hydrolysis and duplex unwinding by Ded1p with 16 bp duplexes containing 25 nt overhang at the 3’ or 5’ terminus, and DNA in the top or bottom strand.

(A) Diagrams for the substrates tested. DNA is in gray, RNA is in black.

(B) Turnover rates ($k_{\text{cat}}$) and enzyme efficiency ($k_{\text{cat}}/K_M$) of ATP hydrolysis stimulated by the substrates depicted in panel A. ATPase assays were performed with 400 nM Ded1p, 2 μM substrates and 2 mM ATP-Mg$_2^+$-EDTA. ATP hydrolysis rates in μM ATP/min were divided by enzyme concentration to obtain $k_{\text{cat}}$. $K_M$ is the Michaelis-Menten constant determined from multiple reactions with different ATP concentrations.

(C) Unwinding rate constants ($k_{\text{unw}}$) for the substrates illustrated in panel A. Values were determined with 1 μM Ded1p, 0.5 nM substrates and 2 mM ATP-Mg$_2^+$ as described previously (Yang and Jankowsky, 2006). Reproduced with permission from the author (Liu, 2010).

In this chapter, we report that ATP hydrolysis on single-stranded overhangs can be uncoupled from unwinding in the presence of low concentrations of ADPNP (~ 10 μM). Ded1p•ADPNP bound to the overhang does not inhibit and in fact facilitates unwinding of the duplex region. These results strongly suggest that distinct loading and unwinding units of Ded1p exist on the single strand and duplex regions respectively, and that the loading unit on
the single-stranded overhang recruits and delivers the unwinding unit to the duplex through oligomerization.

7.2 Results

7.2.1 Differential inhibition by ADPNP suggests distinct loading and unwinding units of Ded1p

To illuminate the mechanism of unwinding by Ded1p, we investigated the effect of the non-hydrolyzable ATP analog, ADPNP, on Ded1p activity with a 16 bp duplex containing a 25 nt single-stranded overhang (Figure 7.2A). We first measured the ATPase activity of Ded1p in the presence of increasing concentrations of ADPNP. The ATP hydrolysis rate decreased as ADPNP was titrated into the reaction. Only half of the activity remained in the presence of ~30 μM ADPNP, and almost 90% inhibition was seen when the concentration of ADPNP was further increased to 2 mM. These results indicate that ADPNP binds to Ded1p with high affinity (K_I ~30 μM) and efficiently competes with ATP. We then examined the effect of ADPNP on ATP-dependent unwinding by Ded1p (Figure 7.2A). Remarkably, the unwinding activity remained unaffected until 10 μM ADPNP, and only then began to decrease. The inhibition of unwinding was significantly less than the inhibition of ATPase at the same ADPNP concentration.

The difference did not reflect technical disparities between unwinding and ATPase assays, as ADP did not inhibit ATPase activity more efficiently than it inhibited unwinding (Figure 7.3). Rather, unwinding was more readily inhibited. Thus, the lack of unwinding
inhibition at \( \leq 10 \, \mu\text{M} \) ADPNP indicates that the ATP hydrolysis inhibited is dispensable for unwinding.

![Figure 7.2](image)

**Figure 7.2 Inhibition of unwinding and ATP hydrolysis activities of Ded1p by ADPNP.**

(A) Unwinding rate constants (open circles) and ATP hydrolysis rates (filled circles) for the 16 bp duplex with 25 nt overhang in the presence of increasing concentrations of ADPNP-Mg\(^{2+}\). The values are normalized according to the respective reactions in the absence of ADPNP. For unwinding reactions, 0.5 nM duplex, 600 nM Ded1p and indicated concentrations of ADPNP-Mg\(^{2+}\) were incubated together for 60 min before 0.5 mM ATP-Mg\(^{2+}\) was added to start the reaction. For ATPase assays, 2 \( \mu\text{M} \) duplex, 400 nM Ded1p and indicated concentrations of ADPNP-Mg\(^{2+}\) were incubated together for 60 min followed by addition of 0.5 mM ATP-Mg\(^{2+}\) to start the reaction. Error bars represent standard deviation of three independent repeats. Fraction of duplex bound by Ded1p under a given ADPNP-Mg\(^{2+}\) concentration was also plotted (open squares). Equilibrium binding was monitored by gel shift assays with 0.5 nM duplex, 600 nM Ded1p and indicated concentrations of ADPNP-Mg\(^{2+}\).

(B) The same unwinding rate constants for the 16 bp duplex with overhang as shown in panel A (open circle) overlaid with ATP hydrolysis rates for the corresponding blunt-ended duplex (filled triangles). ATP hydrolysis rates were measured as in panel A.

We then asked whether the low concentration of ADPNP was sufficient for Ded1p to stably bind the RNA. Gel shift assays were performed with the duplex substrate and increasing concentrations of ADPNP, and the fraction of substrate bound was quantified. Ded1p binding to the substrate was observed with low micromolar concentrations of ADPNP, and was almost complete at 10 \( \mu\text{M} \) ADPNP (Figure 7.2A). Note that this only
reflects Ded1p binding to the single-stranded portion of the RNA substrate, and the single strand alone showed a nearly identical binding profile (Figure 8.3). Together, these results suggest that Ded1p unit(s) bound to the single-stranded overhang turns over ATP but is not the unit that performs the actual duplex unwinding. This notion was additionally supported by the observation that ADP inhibited unwinding more than ATP hydrolysis (Figure 7.3). The results suggest that the unwinding Ded1p unit(s) have a higher affinity for ADP and a lower affinity for ADPNP than the Ded1p unit(s) on ssRNA.

![Figure 7.3](image)

**Figure 7.3 Inhibition of unwinding and ATP hydrolysis activities of Ded1p by ADP.** Unwinding rate constants (open circles) and ATP hydrolysis rates (filled circles) for the 16 bp duplex with 25 nt overhang in the presence of increasing concentrations of ADP-Mg\(^{2+}\), normalized to values in the absence of ADP. For unwinding reactions, 0.5 nM duplex, 600 nM Ded1p and indicated concentrations of ADP-Mg\(^{2+}\) were incubated together for 5 min before 0.5 mM ATP-Mg\(^{2+}\) was added to start the reaction. For ATPase assays, 2 μM duplex, 400 nM Ded1p and indicated concentrations of ADP-Mg\(^{2+}\) were incubated together for 5 min followed by addition of 0.5 mM ATP-Mg\(^{2+}\) to start the reaction. Error bars represent standard deviation of three independent repeats.

To seek evidence for the unwinding unit, we next measured the effect of ADPNP on reactions of a 16 bp duplex with no overhang (Figure 7.2B). ATP hydrolysis was essentially unchanged up to 10 μM ADPNP, and only started to decrease at higher ADPNP concentration. Strikingly, this trend overlaid with ADPNP inhibition of unwinding with the 16 bp - 25 nt overhang duplex (Figure 7.2B), and thus suggests that only ATP hydrolysis on
the duplex portion correlates with unwinding. Ded1p has low affinity for the blunt-ended duplex, precluding measurements of ADPNP inhibition in unwinding reactions. Still, the similar apparent affinities for ADPNP displayed by the blunt-ended duplex in ATPase assay and the overhang-containing duplex in unwinding reaction demonstrate the existence of an unwinding Ded1p unit with lower affinity for ADPNP than the unit(s) on single-stranded overhang. Our data are consistent with a model in which Ded1p bound to single-stranded overhangs hydrolyzes ATP but does not directly participate in duplex unwinding, while ATP binding/hydrolysis by Ded1p bound to duplex regions is required for unwinding.

7.2.2 Ded1p-ADPNP pre-bound on single-stranded overhang allows unwinding of duplex

To directly test the hypothesis that ATP utilization on single-stranded overhang is not required for unwinding, we pre-bound Ded1p to the single-stranded portion under low concentration of ADPNP (50 μM) that allows complete binding (by 2 Ded1p molecules, see Chapter 8) and inhibits ~60% of ATP hydrolysis (Figure 7.2A), and then added ATP to initiate unwinding (Figure 7.4A). Indeed, the duplex with Ded1p-ADPNP pre-bound on the single-stranded overhang was efficiently unwound after ATP addition (Figure 7.4B, Lane 6). The pre-bound Ded1p-ADPNP remained bound to the loading strand, while the non-loading strand was released upon unwinding. Binding of the longer strand but not the shorter strand was not due to preferential binding of Ded1p-ADPNP to the longer strand after unwinding. When Ded1p-ADPNP was pre-loaded onto an unlabeled duplex and ATP was added together with denatured strands from the same duplex label with $^{32}$P, little binding could be seen on the labeled strands (Lane 7). The lack of binding within the 2 min reaction time was consistent with the slow binding kinetics under low ADPNP concentrations (Liu, 2010). The pre-loaded Ded1p-ADPNP that remained bound after unwinding can be removed by
incubation with SDS and EDTA (Lane 9). In a regular ATP-only reaction, Ded1p dissociates from the RNA whether or not SDS and EDTA are included (Lanes 5, 8).

**Figure 7.4 Formation of Ded1p-ADPNP-RNA tight complex does not impede unwinding of duplex.**

(A) Diagram for unwinding reaction with pre-bound Ded1p-ADPNP. The 25 nt overhang-16 bp duplex was labeled with $^{32}$P on both strands (asterisks: radiolabel). 0.5 nM duplex (black lines), 600 nM Ded1p (gray ovals) and 50 μM ADPNP-Mg$^{2+}$ (tan squares) were incubated together for 30 min to allow complete binding of Ded1p-ADPNP to the single-stranded overhang. 2 mM ATP-Mg$^{2+}$ (red triangles) was added to initiate unwinding. Samples were either mixed with 50% glycerol containing 1 μM scavenger RNA (73 nt ssRNA of unrelated sequence) to remove non-specifically bound Ded1p (lanes 3-7 in panel B), or with helicase reaction stop buffer containing 25 mM EDTA and 0.5% SDS to completely remove any bound protein (lanes 8, 9 in panel B).

(B) Native PAGE gel for the reaction described in panel A. (Lane 1) The duplex with $^{32}$P label on both strands (asterisks: radiolabel). (Lane 2) The duplex was heat denatured at 95°C for 2 min, giving rise to two labeled single strands. (Lane 3) Reaction with Ded1p in the absence of ADPNP and ATP. (Lane 4) Reaction with Ded1p and 1 mM ADPNP-Mg$^{2+}$. (Lane 5, 8) Regular unwinding reaction with Ded1p and 2 mM ATP-Mg$^{2+}$ after 2 min. (Lane 6, 9) Ded1p-ADPNP pre-bound to single-stranded overhang followed by ATP-driven unwinding of the duplex, as illustrated in panel A. (Lane 7) Control reaction for Ded1p-RNA binding after unwinding. The reaction was pre-incubated with 50 μM ADPNP-Mg$^{2+}$ as in lane 6, except that an unlabeled duplex was used. ATP was then added together with denatured labeled duplex and incubated for 2 min.

In summary, we demonstrate that a Ded1p-ADPNP complex pre-bound on single-stranded overhang does not inhibit unwinding of the duplex portion, and that the Ded1p-ADPNP complex remains bound to the overhang throughout the unwinding reaction. These results strongly suggest that single-stranded overhangs facilitate unwinding not by
transferring its directly bound Ded1p to the duplex, but by loading of a distinct unit of Ded1p through oligomerization.

7.2.3 Ded1p-ADPNP complexes pre-bound on single-stranded regions facilitate loading of the unwinding unit

The observation in the previous section suggests that the single-stranded overhang functions to load a different unit of Ded1p onto the duplex through oligomerization. To gain further support for this model, we pre-bound Ded1p-ADPNP to the overhang portion and assayed for unwinding over a range of Ded1p concentrations. Unwinding with Ded1p-ADPNP unit(s) pre-bound to the overhang was faster than without pre-bound units (Figure 7.5), consistent with the oligomerization mechanism.

![Figure 7.5 Ded1p-ADPNP units pre-bound on single-stranded overhang aid loading of unwinding Ded1p units.](image)

Unwinding rate constants with (filled circles) and without (open circles) pre-loaded Ded1p-ADPNP measured over a range a Ded1p concentration. Reactions were performed as illustrated in Figure 7.4 for the Ded1p concentrations indicated, with or without 20 μM ADPNP-Mg²⁺. Error bars represent standard deviation of two independent experiments. Curves represent the best fit to Hill equation, $k_{unw} = k_{unw,max} [\text{Ded1p}]^n / (K_{1/2}^n + [\text{Ded1p}]^n)$, where $K_{1/2}$ represents functional affinity Ded1p, n is Hill coefficient. For reactions without pre-bound Ded1p-ADPNP, $k_{unw,max} = 3.2 \pm 1.0 \text{ min}^{-1}$, $K_{1/2} = 319 \pm 139 \text{ nM}$, $n = 1.6 \pm 0.5$; or $k_{unw,max} = 8.1 \pm 4.5 \text{ min}^{-1}$, $K_{1/2} = 1403 \pm 1038 \text{ nM}$ when n was set to 1. For reactions with pre-bound Ded1p-ADPNP, $k_{unw,max} = 4.1 \pm 1.5 \text{ min}^{-1}$, $K_{1/2} = 269 \pm 212 \text{ nM}$, $n = 1.0 \pm 0.4$. 
7.3 Discussion

Our results show that Ded1p units bound to a single-stranded overhang do not perform the actual unwinding of the duplex. Instead, they facilitate loading of a distinct unwinding unit to the duplex (Figure 7.6). Ded1p can be immobilized on the single-stranded overhang and still facilitate unwinding. Therefore, transfer of Ded1p from single strand to the duplex is at least not necessary. While we favor a mechanism in which Ded1p units bound to the overhang help to load the unwinding unit onto the duplex region, the current data are equally consistent with a situation where Ded1p units bound to the overhang stabilize existing Ded1p binding to the duplex, before or during unwinding. In either case, our data suggest that Ded1p functions as an oligomer during the unwinding reaction (Figure 7.6).

Figure 7.6 Model for Ded1p loading to duplex regions aided by single-stranded overhangs.
An RNA substrate contains duplex and single-stranded regions in proximity to one another. The loading unit(s) (cyan circles) binds to single-stranded regions dependent on ATP (red rectangles). ATP hydrolysis (yellow rectangles indicate ADP) followed by product release leads to dissociation of the loading unit(s) from the ssRNA. An unwinding unit (beige circles) is recruited by the loading units and directly binds to duplex regions. After local strand separation by the unwinding unit, ATP hydrolysis and product release allows dissociation of the unwinding unit. Chapter 8 presents evidence for the number of Ded1p molecules on single-stranded regions. The number of Ded1p monomers in an unwinding unit is unknown. The order of ATP hydrolysis among Ded1p units is not considered in this diagram. ATP hydrolysis before complete strand separation also leads dissociation of the units, but is omitted for clarity.
The non-hydrolyzable ATP analog ADPNP does not support unwinding by Ded1p, but allows Ded1p to stably clamp on a single-stranded overhang and oligomerize with additional units of Ded1p that initiate unwinding within the duplex. It is not clear whether ADPNP mimics an on-pathway state during ATP hydrolysis. The ability to tightly lock Ded1p on single-stranded RNA has also been observed for the transition state ATP analogs ADP-AlF₄ and ADP-MgFₓ, and the ground state analog ADP-BeFₓ (Liu et al., 2008; Liu, 2010). Crystal structures of the DEAD-box protein Mss116p in complex with single-stranded RNA and ATP analogs ADPNP, ADP-AlF₄ and ADP-BeF₃ are highly superimposable (Del Campo and Lambowitz, 2009). However, only the ground state ATP analog ADP-BeFₓ is sufficient for a single round of duplex unwinding (Liu et al., 2008). Together, these results suggest that Ded1p binds to ssRNA in a similar conformation for the different ATP analogs. Ded1p in this conformation is capable of oligomerizing with additional unit(s) of Ded1p, which is capable of duplex unwinding only when ATP or ADP-BeFₓ is present.

ATP hydrolysis by Ded1p on single-stranded RNA is not required either by the loading unit (replaced by ADPNP), or by the unwinding unit (replaced by ADP-BeFₓ). ATP hydrolysis is essential for enzyme turnover, as all the tested ATP analogs keep Ded1p on single-stranded RNA for hrs (Table 8.2), and thus does not allow unwinding under steady-state conditions (Liu et al., 2008). It is yet possible that ATP hydrolysis offers additional advantages to the loading and unwinding units of Ded1p. The extant data, however, imply that fast ATP hydrolysis on single-stranded overhangs may lead to too frequent dissociations without loading of unwinding units onto the duplex, or without complete strand separation (Figure 7.6). Interestingly, the DEAD-box protein Mss116p, a faster ATPase than Ded1p (Fei Liu, unpublished observation), displays higher affinity for structured RNA than Ded1p.
(Mohr et al., 2008; Del Campo et al., 2009), which would alleviate the potential
disadvantage of a fast ATPase.
Chapter 8

ATP analog-dependent binding of multiple Ded1p molecules on single-stranded RNA

8.1 Introduction

In Chapter 7, we showed that RNA duplex unwinding by the DEAD-box protein Ded1p involves multiple enzyme units. Ded1p unit(s) bound to single-stranded RNA facilitates loading of additional unit(s) of Ded1p to the adjacent duplex, where unwinding is initiated (Yang and Jankowsky, 2006). In this chapter, we seek to determine how many molecules of Ded1p are bound on single-stranded RNAs, and to elucidate whether and how Ded1p bound on single-stranded RNAs recruit additional unit(s) of Ded1p in different states of ATP hydrolysis.

8.2 Results

8.2.1 Determining the number of Ded1p molecules bound on RNA by sucrose gradient sedimentation

To investigate how many molecules of Ded1p are bound to a given RNA substrate, a $^{32}$P-labelled RNA substrate was incubated together with saturating concentrations of Ded1p and ATP analog (ADPNP, ADP-AlF$_4$ or ADP-BeF$_x$), and the sample was subjected to sucrose gradient sedimentation. Three well-characterized proteins were used as size

150
standards: ovalbumin monomer (45 kDa), aldolase dimer (149 kDa) and catalase tetramer (240 kDa).

Peak fraction numbers of the size standards were fit to equation \( F = m(MW)^{2/3} \), where \( F \) is the peak fraction number, \( m \) is a constant dependent on experimental conditions, \( MW \) is the molecular weight (Schachman, 1959; Martin and Ames, 1961). The molecular weight of the species in the sample was then calculated from the fit. Because RNAs have higher density (lower partial specific volume) than proteins (Voss and Gerstein, 2005), molecular weight of free RNAs estimated according to the size standards is higher than its actual molecular weight. For example, the 16 bp duplex with 25 nt overhang has a molecular weight of 19 kDa, but sediments together with a 45 kDa protein (Figure 8.1).

![Figure 8.1 Ded1p-RNA binding reactions without scavenger RNA show heterogeneous complexes after sucrose gradient sedimentation.](image)

A \(^{32}\)P-labeled 16 bp RNA duplex with 25 nt overhang was incubated with (red circles) or without (black circles) Ded1p and ADPNP, and then loaded immediately onto a 6-40% sucrose gradient. Samples after ultra-centrifugation were fractionated, and subjected to scintillation counting to measure the amount of the RNA in each fraction. Curves represent interpolations of the data points. Dashed lines indicate peak fraction numbers of proteins used as molecular weight markers sedimented under
identical conditions. The proteins are ovalbumin monomer (45 kDa), aldolase tetramer (149 kDa) and catalase tetramer (240 kDa).

(A) 1 nM duplex was incubated with 700 nM Ded1p and 1 mM ADPNP-Mg²⁺ for 5 min.
(B) 1 nM duplex was incubated with 700 nM Ded1p and 0.22 mM ADPNP-Mg²⁺ for 40 min.

The 66.4 kDa Ded1p was detected in fractions consistent with its molecular weight as a monomer (data not shown). Any larger molecular weight complexes are thereby interpreted as one or more Ded1p molecules bound to RNA.

In order to only measure stable binding by Ded1p, we included a large excess of scavenger RNA before the Ded1p-RNA binding reaction was layered onto the sucrose gradient. When scavenger RNA was not included, no distinct peak could be observed (Figure 8.1). Addition of scavenger RNA removed excess and not stably bound Ded1p molecules, and a clear peak emerged (Figure 8.3B). In gel shift assays, incubation with scavenger RNA also removed heterogeneously associated Ded1p (data not shown). Under such conditions, however, Ded1p binding to the 16 bp duplex with 25 nt overhang showed two bound species with the same dependence on ADPNP concentration as the 25 nt overhang alone (Figure 8.2). This observation suggests that duplex regions do not notably contribute to Ded1p binding when scavenger is included, and only Ded1p molecules directly or indirectly bound to single-stranded regions can be detected.
Figure 8.2 Duplex regions do not contribute to Ded1p binding in gel shift assays. Ded1p binding to a 16 bp duplex with 25 nt single-stranded overhang 3’ to the duplex (A) and a 25 nt single strand with the same sequence as the overhang (B). 0.5 nM 32P-labeled RNA was incubated with 800 nM Ded1p and 1 to 500 μM ADPNP-Mg2+ for 60 min. Ded1p-bound species were separated from free RNA by native PAGE (top panels). Results were quantified in ImageQuant software and plotted against ADPNP concentration (bottom panels). Red circles: lower bound band. Green circles: higher bound band. Blue circles: the two Ded1p-bound species quantified together.

8.2.2 In the presence of ADPNP, multiple molecules of Ded1p bind to single-stranded overhang, each occupying ~10 nt

To determine the number of Ded1p molecules bound to RNA substrates in the presence of ADPNP, a binding reaction was performed with Ded1p and a 16 bp duplex with 25 nt overhang and analyzed by sucrose gradient sedimentation. The RNA alone peaked at 45 kDa (Figure 8.3B). After incubation with saturating concentrations of Ded1p and the ATP analog ADPNP, the RNA peak was shifted to 174.3 ± 0.9 kDa (Figure 8.3B, middle panel, Table 8.1), consistent with two molecules of Ded1p bound (66.4 kDa per molecule). Similarly, gel shift assays revealed two bound species as ADPNP was titrated into the reaction (Figure 8.3B, right panel). Assuming no partial binding of any molecule on the
single-stranded region, the 25 nt overhang is enough for 2 Ded1p molecules but not for 3. Thus, each Ded1p molecule occupies 25/3 - 25/2 nt, equivalent to 9-12 nt.

Figure 8.3 In the presence of ADPNP, multiple molecules of Ded1p bind to single-stranded overhang, each occupying 9-11 nt. (Left panels) Diagrams of 16 bp RNA duplexes with single-stranded overhangs of 35 nt (A), 25 nt (B), 15 nt (C) and 10 nt (D). (Middle panels) Distribution of RNA alone (black circles) and RNA with Ded1p and ADPNP (red circles) after sucrose gradient fractionation. Binding reactions were performed with 1 nM 32P-labeled RNA, 1.1 μM Ded1p and 1 mM ADPNP-Mg2+ for 60 min. 1 μM of a 73 nt single-stranded RNA of irrelevant sequence was added as scavenger, before the sample was layered onto a 6-40% sucrose gradient. After ultra-centrifugation, the sample was fractionated and subjected to scintillation counting to measure the amount of the 32P-labeled RNA in each fraction. Curves represent best fits to Gaussian distribution, with peak positions listed in Table 8.1. When more than one peaks are present in the Ded1p-ADPNP sample (panels C, D), pink traces indicate individual Gaussian distributions, while red curves show sum of the individual distributions. Dashed lines indicate peak fraction numbers of proteins sedimented under identical condition as molecular weight markers, as in Figure 8.1. (Right panels) Gel shift assays for Ded1p-ADPNP binding to the duplexes. 0.5 nM 32P-labeled RNA, 800 nM Ded1p and increasing concentrations of ADPNP-Mg2+ were incubated together for 60 min. Ded1p-bound species and free RNA were separated by native PAGE. Schematics on the left indicate number of Ded1p molecules (cyan ovals) bound.
Table 8.1 Molecular weight of Ded1p-ADPNP-RNA complexes shown in Figure 8.2.

<table>
<thead>
<tr>
<th>RNA</th>
<th># Ded1p molecule</th>
<th>Peak fraction</th>
<th>Expected MW (kDa)</th>
<th>Measured MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 bp with 35 nt oh</td>
<td>3</td>
<td>43.3 ± 0.3</td>
<td>216.0</td>
<td>249.6 ± 2.6</td>
</tr>
<tr>
<td>16 bp with 25 nt oh</td>
<td>2</td>
<td>29.9 ± 0.1</td>
<td>146.3</td>
<td>174.3 ± 0.9</td>
</tr>
<tr>
<td>16 bp with 15 nt oh</td>
<td>2</td>
<td>31.5 ± 0.4</td>
<td>143.0</td>
<td>188.5 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21.0 ± 0.2</td>
<td>76.6</td>
<td>102.6 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.9 ± 0*</td>
<td>10.2</td>
<td>38.4 ± 0.1</td>
</tr>
<tr>
<td>16 bp with 10 nt oh</td>
<td>1</td>
<td>23.0 ± 0.3</td>
<td>75.0</td>
<td>114.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>10.7 ± 0.1</td>
<td>8.6</td>
<td>36.3 ± 0.5</td>
</tr>
</tbody>
</table>

Reactions and data analysis performed as shown in Figure 8.3. The asterisk for the 16 bp duplex with 15 nt overhang indicates that the free RNA peak was fixed at fraction 10.9.

To further analyze the binding site size of Ded1p, experiments were carried out for a series of 16 bp duplexes with different length of single-stranded overhangs. When the overhang is 35 nt, the RNA alone sedimented slightly higher than 45 kDa (Figure 8.3A). Upon Ded1p-ADPNP binding, the RNA peak was shifted to 249.6 ± 2.6 kDa (Figure 8.3A, middle panel, Table 8.1), corresponding to a complex with three molecules of Ded1p bound. This was again consistent with gel shift results (Figure 8.3A, right panel). The binding of three Ded1p molecules translates to a binding site of 35/4-35/3 nt, or 9-11 nt, which perfectly overlays with the 9-12 nt binding site determined from the duplex with 25 nt overhang. For a duplex with 15 nt overhang, a major Ded1p-ADPNP-bound peak was observed at 102.6 ± 1.5 kDa, consistent with a single Ded1p molecule bound (Figure 8.3C, Table 8.1). In addition, two minor peaks were detected at 38.4 ± 0.1 and 188.5 ± 3.6 kDa, corresponding to free RNA and to two Ded1p molecules respectively (Figure 8.3C, Table 8.1). The major peak with a monomer bound is consistent the 9-11 nt binding site size derived from results with 35 and 25 nt overhangs (Figure 8.3A, B, Table 8.1); the minor peak with two Ded1p molecules implies binding to an incomplete site stabilized by protein-protein interactions (see Sections 8.2.4-8.3). Both of the bound species were observed in gel shift assays (Figure 8.3C, right
panel). The presence of the free RNA fraction is consistent with the faster dissociation from the shorter overhangs (Table 8.2, 25 nt vs. 10 nt). We further verified the binding site size with a duplex containing only 10 nt single-stranded overhang. Gel shift assays showed a single bound species (Figure 8.3D), the half-life of which is only about 2 hrs (Table 8.2). Consequently, the majority of the RNA was seen in free fractions after 15 hrs of centrifugation in a sucrose gradient (Figure 8.2D). Still, a shoulder next to the free RNA peak corresponded to a single Ded1p molecule bound. We concluded that the binding site size of one Ded1p molecule in the presence of ADPNP is approximately 10 nt.

### Table 8.2 Off-rate and half-life of Ded1p-ADPNP-RNA complexes measured by gel shift assay.

<table>
<thead>
<tr>
<th>RNA</th>
<th>$k_{off}$ (10$^{-5}$ • min$^{-1}$)</th>
<th>$t_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 bp with 25 nt oh</td>
<td>37.0 ± 2.3</td>
<td>31.2 ± 1.9</td>
</tr>
<tr>
<td>16 bp with 10 nt oh</td>
<td>495 ± 14</td>
<td>2.33 ± 0.07</td>
</tr>
<tr>
<td>25 nt single strand</td>
<td>88.3 ± 5.3</td>
<td>13.1 ± 0.8</td>
</tr>
<tr>
<td>10 nt single strand</td>
<td>565 ± 24</td>
<td>2.04 ± 0.09</td>
</tr>
</tbody>
</table>

Equilibrium binding reactions as shown in Figure 8.2 were challenged with 1 μM of a 73 nt scavenger RNA (see Chapter 10 for details). Dissociation of Ded1p from RNA substrates was quantified and fit to a single exponential to determine the dissociation rate constants ($k_{off}$) and half-life ($t_{1/2}$) of the complexes.

### 8.2.3 Alkaline-hydrolyzed ladder confirms the ~10 nt binding site of Ded1p-ADPNP on single-stranded RNA

In order to systematically examine the binding of Ded1p-ADPNP to RNAs of different length, and to confirm that only Ded1p molecules bound to single-stranded RNA are detected, we developed an assay that measures binding at single-nucleotide resolution. A 36 nt poly(U) RNA was subjected to alkaline hydrolysis to generate a uniformly hydrolyzed ladder ranging from 1-36 nt. The pool of single strands was incubated with Ded1p and ADPNP as described in Session 8.2.1, and fractions after sucrose gradient sedimentation
were analyzed by denaturing PAGE at single nucleotide resolution (Figure 8.4B). The method allowed us to determine how many Ded1p molecules were bound to each RNA between 1 and 36 nt at the same time.

Figure 8.4 Ded1p-ADPNP binding to poly(U) ladder. A 5'-32P-labeled poly(U) RNA 36 nt in length was subjected to limited alkaline hydrolysis to produce a ladder of 1-36 nt single strand. The RNA ladder was then used for Ded1p binding and sucrose gradient sedimentation as described in Figure 8.2, with 2 nM 32P-labeled RNA, 867 nM Ded1p and 0.5 mM ADPNP-Mg2+.
(A) The RNA ladder alone was fractionated after sucrose gradient sedimentation. Samples from the fractions indicated were analyzed by denaturing PAGE to single-nucleotide resolution.

(B) The RNA ladder with Ded1p-ADPNP bound was fractionated and analyzed by denaturing PAGE. Arrows to the right mark ssRNAs plotted in panel C.

(C) Distributions of 35 nt, 25 nt, 15 nt and 10 nt single strand quantified from the gel shown in panel B. Curves represent best fits to Gaussian distribution. When more than one peaks are present, dashed traces indicate individual Gaussian distributions, while solid curves show sum of the individual distributions.

(D) Contour plot showing distribution of individual length of poly(U) in each fraction. For each lane in panel B, signal from individual poly(U) length (U_i, i = 2...36) was divided by the total signal from all bands \( \Sigma(U_2...U_{36}) \). The data were imported into Mathematica software to generate the contour plot. The contour lines are 1, 2.8 (average if each length is equally represented), 4, 6, 10%. The number of Ded1p molecules bound is marked above the plot and illustrated in schematics to the right of the plot. Dashed lines indicate gradient fractions plotted in panel E.

(E) Distribution of individual length of poly(U) in representative gradient fractions. Normalized signals from gradient fractions corresponding to free RNA (fraction 4, blue circles), 1 Ded1p molecule bound (fraction 21, magenta circles), 2 Ded1p molecules bound (fraction 30, green circles), and 3 Ded1p molecules bound (fraction 36, red circles) as shown in panel D were plotted against RNA length. The dash line highlights the length cutoff for Ded1p binding.

Binding by Ded1p-ADPNP gave rise to a distinct distribution pattern of the ladder RNA (Figure 8.4A). RNAs shorter than 10 nt were only found in fractions corresponding to free RNA. RNAs 10-20 nt in length appeared in fractions that are consistent with one Ded1p molecule bound. Longer RNAs were observed in higher molecular weight fractions as more Ded1p molecules were able to bind. Such separation of RNAs according to their length was not seen with the ladder RNA alone (Figure 8.4B), and is therefore dependent on Ded1p binding.

Bands representing 35, 25, 15 and 10 nt single strands were complexed with the same number of Ded1p molecules as duplexes with corresponding length of overhang (Figure 8.2, 8.4C). These results demonstrate that only Ded1p molecules bound to single-stranded regions are detected with our method, and that Ded1p binding occurs regardless of the sequence of RNA used.

We further analyzed the data with the poly(U) ladder by quantifying the distribution of RNA length in each gradient fraction. For each length of poly(U), the signal in each gradient fraction is divided by the total signal of poly(U) in the corresponding fraction, in
exact, $U_i / \Sigma (U_2 \ldots U_{36})$, $i = 2 \ldots 36$ (for $U_1$, signals from different lanes appeared merged on the PAGE gel). Results from the quantification were shown in a contour plot to show the distribution of different lengths of RNAs in the sucrose gradient (Figure 8.4D), and in a scatter plot to show the distribution in representative gradient fractions (Figure 8.4E). We observed a clear transition at ~10 nt from free RNA to one Ded1p molecule bound (Figure 8.4D, E). Resolution declined for longer RNA, yet the results are still consistent with a binding site of ~10 nt per Ded1p molecule.

As it is possible that ADPNP dissociates from Ded1p during centrifugation, leading to changes in the binding pattern, we performed the same binding reaction with the poly(U) ladder and centrifuged the sample in a sucrose gradient containing the same concentration of ADPNP as the binding reaction (Figure 8.5). The results were similar to the reaction with ADPNP not present in the sucrose gradient (Figure 8.4, 8.5). Taken together, each Ded1p molecule binds approximately 10 nt single-stranded RNA in the presence of ADPNP.
Figure 8.5 Ded1p-ADPNP binding to poly(U) ladder with ADPNP both in reaction and in the sucrose gradient.

Binding reaction as in Figure 8.4, with 0.5 mM ADPNP-Mg$^{2+}$ in the sucrose gradient. 

(A) The RNA ladder with Ded1p-ADPNP bound was fractionated and analyzed by denaturing PAGE. Arrows to the right indicate ssRNAs plotted in panel B.

(B) Distributions of 35 nt, 25 nt, 15 nt and 10 nt single strand quantified from the gel shown in panel C. Curves represent best fits to Gaussian distribution. When more than one peaks are present, dashed traces indicate individual Gaussian distributions, while solid curves show sum of the individual distributions.

(C) Contour plot showing distribution of individual length of poly(U) in each fraction. For each lane in panel A, signal from individual poly(U) length ($U_i$, i = 2…36) was divided by the total signal from all bands ($\Sigma(U_2…U_{36})$). The data were imported into Mathematica software to generate the contour plot. The contour lines are 1, 2.8 (average if each length is equally represented), 4, 6, 10%. The number of
Ded1p molecules bound is marked above the plot and illustrated in schematics to the right of the plot. Dashed lines indicate gradient fractions plotted in panel D.

**(D)** Distribution of individual length of poly(U) in representative gradient fractions. Normalized signals from gradient fractions corresponding to free RNA (fraction 4, blue circles), 1 Ded1p molecule bound (fraction 21, magenta circles), 2 Ded1p molecules bound (fraction 30, green circles), and 3 Ded1p molecules bound (fraction 36, red circles) as shown in panel C were plotted against RNA length. The dash line highlights the length cutoff for Ded1p binding.

### 8.2.4 In the presence of ADP-BeF₆⁺, more Ded1p molecules are bound to single-stranded RNA than in the presence of ADP-BeF₆⁺

While the non-hydrolyzable ATP analog ADPNP enables stable binding of Ded1p on single-stranded RNA, it does not allow unwinding of the duplex region (Liu *et al.*, 2008). We asked whether the ground state analog ADP-BeF₆⁺ that does support duplex unwinding shows a different mode of Ded1p binding on single-stranded RNA. On a 25 nt single strand, three instead of two Ded1p molecules were observed (Figure 8.6A). If all three Ded1p molecules bound the RNA with the same binding site size, the binding site would be 25/4-25/3 nt, equivalent to 7-8 nt. Alternatively, two Ded1p molecules might bind the RNA as seen with ADPNP, while a third Ded1p molecule associates with the RNA-bound Ded1p and does not or only partially contacts the RNA.

When we examined binding to a 10 nt single strand, two peaks were observed dependent on the concentration of ADP-BeF₆⁺ (Figure 8.6B). The peaks corresponded to one and two (two instead of three, explained in the next paragraph with Figure 8.7) Ded1p molecules bound. Two Ded1p molecules bound on a 10 nt RNA would mean 4-5 nt (10/3-10/2 nt) per molecule if only intact binding sites were considered, inconsistent with results with the 25 nt single strand. If additional Ded1p molecule(s) could associate with RNA-bound Ded1p, however, the binding site size would still be ~10 nt for one Ded1p molecule, while a second Ded1p molecule would be bound through protein-protein interactions.
To test the two binding scenarios, we examined whether Ded1p-ADP-BeFx could bind to a 5 nt single strand (Figure 8.6C). When reactions were performed under conditions identical to reactions with the 25 and 10 nt single strands, only free RNA was seen, arguing against a 4-5 nt binding site size. When ADP-BeFx was present across the entire sucrose gradient, however, a single Ded1p molecule was bound to the 5 nt RNA. As Ded1p has been shown to stably bind ADP-BeFx and Mg2+ when bound on single-stranded RNA (10 nt and
longer) (Liu, 2010), we favor the interpretation that this single Ded1p molecule on 5 nt single strand does not represent a fully locked-on conformation, but contains solvent-exposed ADP-BeF$_x$ that dissociates during the centrifugation.

To better understand the binding of Ded1p-ADP-BeF$_x$ to RNAs with different length, we again performed binding reactions with alkaline-hydrolyzed poly(U) ladder, with ADP-BeF$_x$ present in the entire sucrose gradient. Denaturing PAGE displayed distinct grouping of different length of RNA (Figure 8.7A). Binding to 25, 10 and 5 nt single strands showed results identical to those when they were individually assayed (Figure 8.6, 8.7B). No binding was observed for the 4 nt single strand, indicating 5 nt as the minimum binding site (Figure 8.7B).

When the distribution of RNA length in each gradient fraction was quantified and plotted, we observed two clear transitions: one at ~5 nt, from free to one Ded1p molecule bound, the other at ~10 nt, from one to two Ded1p molecules bound (Figure 8.7C, D). While these results are perfectly consistent with a binding site of 5 nt per Ded1p molecule, RNAs of 10-20 nt sedimented as a continuous group with two Ded1p molecules bound (Figure 8.7A, C, D). Furthermore, for RNAs of 10-20 nt, the shorter RNAs appeared in higher molecular weight fractions, almost assuming as three Ded1p molecules bound instead of two (e.g. on 10 nt RNA, Figure 8.6B, 8.7B). This phenomenon may be better explained by a scenario where
Figure 8.7 Ded1p-ADP-BeF$_x$ binding to poly(U) ladder, with ADP-BeF$_x$ present throughout the sucrose gradient.

A 5' $^{32}$P-labeled poly(U) ladder (1-36 nt) was incubated with Ded1p and ADP-BeF$_x$, as described in Figure 8.4 for ADPNP, except that 0.5 mM ADP-Mg$^{2+}$, 0.75 mM BeF$_2$ and 3.75 mM NaF (1 : 1.5 : 7.5) were present both in the binding reaction and in the sucrose gradient.

(A) The RNA ladder with Ded1p-ADP-BeF$_x$ bound was fractionated after sucrose gradient sedimentation and analyzed by denaturing PAGE. Arrows to the right indicate ssRNAs plotted in panel B.

(B) Distributions of 25 nt, 10 nt, 5 nt and 4 nt single strand quantified from the gel shown in panel A. Curves represent best fits to Gaussian distribution. When more than one peaks are present, dashed traces indicate individual Gaussian distributions, while solid curves show sum of the individual distributions.

(C) Contour plot showing distribution of individual length of poly(U) in each fraction. For each lane in panel A, signal from individual poly(U) length ($U_i$, $i = 2 \ldots 36$) was divided by the total signal from all
bands ($\Sigma(U_2\ldots U_{36})$). The data were imported into Mathematica software to generate the contour plot. The contour lines are 1, 2.8 (average if each length is equally represented), 4, 6, 10%. The number of Ded1p molecules bound is marked above the plot and illustrated in schematics to the right of the plot. Dashed lines indicate gradient fractions plotted in panel D.

**D** Distribution of individual length of poly(U) in representative gradient fractions. Normalized signals from gradient fractions corresponding to free RNA (fraction 4, blue circles), 1 Ded1p molecule bound (fraction 21, magenta circles), 2 Ded1p molecules bound (fraction 33, green circles), 3 Ded1p molecules bound (fraction 38, red circles), and 4 Ded1p molecules bound (fraction 46, yellow circles) as shown in panel C were plotted against RNA length. Dashed lines highlight length cutoffs for Ded1p binding.

one Ded1p molecule requires 10 nt RNA for full binding, only upon which can a second Ded1p molecule associates with the RNA-bound molecule, at the same time grasping additional RNA for itself. The apparently higher molecular weight with the shorter RNAs may reflect a flexible conformation of Ded1p that is gradually restrained as a full binding site on RNA emerges for the second Ded1p molecule. RNAs beyond 20 nt recruited one Ded1p molecule about every 5 nt, resulting either from simple binding to 5 nt or from association with RNA-bound Ded1p. Taken together, our observations at least demonstrated that oligomerization of Ded1p on RNA is influenced by the state of ATP hydrolysis. Higher oligomers form with the ground state analog ADP-BeF$_x$, but not with the non-hydrolyzable analog ADPNP.

**8.2.5** *Ded1p-ADP-AlF$_4$ binds ssRNA similar to Ded1p-ADPNP when the analog is present only in the reaction, while similar to Ded1p-ADP-BeF$_x$ when present both in the reaction and in the sucrose gradient*

Having seen that ADP-BeF$_x$ leads to association of more molecules of Ded1p with RNA than ADPNP, we next asked how the transition state analog ADP-AlF$_4$ would affect binding of Ded1p. When gel shift assays were performed with increasing Ded1p concentrations, two bound species were observed on the 25 nt single strand, and one bound species was observed on the 10 nt single strand, similar to results with ADPNP, not with
ADP-BeF₆ (Figure 8.8). On a 5 nt single strand, however, binding of Ded1p could be detected, although not to the same extent as with ADP-BeF₆ (Figure 8.8).

We then examined RNA binding by Ded1p-ADP-AlF₄ using sucrose gradient sedimentation. When ADP-AlF₄ was only included in the binding reaction, two Ded1p molecules were bound on a 25 nt single strand, consistent with gel shift results (Figure 8.8B, 8.9A). Binding was incomplete due to the low concentration of ADP-AlF₄ used (Figure 8.9A). However, when ADP-AlF₄ was also present across the sucrose gradient, three Ded1p

![Figure 8.8 Ded1p binding to single-stranded RNAs in the presence of ADPNP, ADP-AlF₄ and ADP-BeF₆.](image)

0.5 nM of 25 nt (top panels), 10 nt (middle panels) or 5 nt (bottom panels) single-stranded RNA was incubated with the indicated ATP analog and increasing concentration of Ded1p (0, 25, 150, 300, 800 nM) and analyzed by native PAGE.

(A) Binding reactions with 0.5 mM ADPNP-Mg²⁺, incubated for 15 min for the 25 nt RNA (top panel), 60 min for the 10 and the 5 nt RNAs (middle and bottom panels).

(B) Binding reactions with 0.5 mM ADP-Mg²⁺, 2.5 mM AlF₃ and 12.5 mM NaF (1 : 5 : 25), incubated for 15 min for the 25 nt RNA (top panel), 60 min for the 10 and the 5 nt RNAs (middle and bottom panels).

(C) Binding reactions with 0.5 mM ADP-Mg²⁺, 2.5 mM BeF₆ and 12.5 mM NaF (1 : 5 : 25), incubated for 15 min for the 25 nt RNA (top panel), 30 min for the 10 and the 5 nt RNAs (middle and bottom panels).
molecules were bound to the 25 nt ssRNA (Figure 8.9B), reminiscent of results with ADP-BeF₆ (Figure 8.6A). These results suggest that two Ded1p molecules are stably bound on the 25 nt single strand, the third Ded1p molecule only joins when its relatively open ATP site was occupied by ADP-AlF₄.

**Figure 8.9** Oligomerization state of Ded1p-ADP-AlF₄ is similar to ADPNP when ADP-AlF₄ is absent from the sucrose gradient, while it is similar to ADP-BeF₆ when ADP-AlF₄ is present in the gradient.

Sucrose gradients showing Ded1p-ADP-AlF₄ binding on a 25 nt single-stranded RNA (A, B), a 10 nt single-stranded RNA (C) and a 5 nt single-stranded RNA (D). Blue circles (A): 867 nM Ded1p incubated with 50 nM ³²P-labeled RNA and 0.5 mM ADP-Mg²⁺, 0.75 mM AlF₃, 3.75 mM NaF (1 : 1.5 : 7.5) for 60 min, diluted 50-fold with reaction buffer, and mixed with 1 μM scavenger RNA before loading onto the sucrose gradient. Light blue triangles (B): 867 nM Ded1p incubated with 1 nM ³²P-labeled RNA and 0.5 mM ADP-Mg²⁺, 2.5 mM AlF₃, 7.5 mM NaF (1 : 5 : 15) for 60 min, and then mixed with 1 μM scavenger RNA. The sucrose gradient contains the same concentrations of ADP-Mg²⁺, AlF₃ and NaF (1 : 5 : 15). Green triangles (C, D): 867 nM Ded1p incubated with 1 nM ³²P-labeled RNA and 0.5 mM ADP-Mg²⁺, 2.5 mM AlF₃, 12.5 mM NaF (1 : 5 : 25) for 60 min, and then mixed with 1 μM scavenger RNA. The sucrose gradient contains the same concentrations of ADP-Mg²⁺, BeF₂ and NaF (1 : 5 : 25). After ultracentrifugation, the sample was fractionated and subjected to scintillation counting to measure the amount of the ³²P-labeled RNA in each fraction. The fraction size is the same as in Figure 8.2. Dashed lines mark peak positions of proteins used as molecular weight markers. Curves represent best fits to Gaussian distribution. When more than one peaks are present, dashed traces indicate individual Gaussian distributions, while solid curves show sum of the individual distributions.
We note that ADP-BeFx does not need to be present in the gradient for association of additional Ded1p molecules on 25 and 10 nt RNAs. Thus, ADP-BeFx may be more tightly bound to non-RNA-bound Ded1p molecules than ADP-AlF$_4$. This idea predicts that if ADP-AlF$_4$ is present throughout the sucrose gradient, it will promote Ded1p-Ded1p interactions just as ADP-BeFx does. Indeed, we detected binding of a second Ded1p molecule to the 10 nt single strand when ADP-AlF$_4$ is present throughout the sucrose gradient (Figure 8.9C, two Ded1p molecules instead of three, as with ADP-BeFx). In contrast, gel shift assays with increasing Ded1p concentrations only showed a single Ded1p-bound form (Figure 8.8B). Binding to the 5 nt single strand was also detectable (Figure 8.9D), as seen with ADP-BeFx (Figure 8.6C).

To further test whether ADP-AlF$_4$ enables Ded1p oligomerization similar to ADP-BeFx when it is included both in the binding reaction and during the sedimentation, we carried out binding reactions with the poly(U) ladder. The results were essentially the same as those with ADP-BeFx (cf. Figures 8.7, 8.10). Bands for 25, 10 and 5 nt single strands showed results identical to when they were individually assayed (Figure 8.9B-D, 8.10B). Little binding was observed for the 4 nt single strand, indicating 5 nt as the minimal binding site (Figure 8.10B). When the distribution of RNA length in each gradient fraction was quantified and plotted, we observed two clear transitions at ~5 nt from free to one Ded1p molecule bound, and at ~10 nt from one to two Ded1p molecules bound (Figure 8.10C, D). RNAs of 10-20 nt again sedimented as a continuous group, with the shorter RNAs appeared in apparently higher molecular weight fractions (Figure 8.10A, C). RNAs beyond 20 nt recruited one Ded1p molecule about every 5 nt. Collectively, Ded1p-ADP-AlF$_4$ binds to
single-stranded RNA in a way highly similar to Ded1p-ADP-BeFx when the analog is present in the sucrose gradient.

Figure 8.10 Ded1p-ADP-AlF₄⁻ binding to poly(U) ladder when ADP-AlF₄⁻ is present in the gradient.
A 5’-³²P-labeled poly(U) ladder (1-36 nt) was incubated with Ded1p and ADP-AlF₄⁻ as described in Figure 8.4 for ADPNP, except that 0.5 mM ADP-AlF₂⁻Mg²⁺, 2.5 mM AlF₃⁻ and 12.5 mM NaF (1 : 5 : 25) were present both in the binding reaction and in the sucrose gradient.

(A) The RNA ladder with Ded1p-ADP-AlF₄⁻ bound was fractionated after sucrose gradient sedimentation and analyzed by denaturing PAGE. Arrows to the right indicate ssRNAs plotted in panel B.
(B) Distributions of 25 nt, 10 nt, 5 nt and 4 nt single strand quantified from the gel shown in panel A. Curves represent best fits to Gaussian distribution. When more than one peaks are present, dashed traces indicate individual Gaussian distributions, while solid curves show sum of the individual distributions.

(C) Contour plot showing distribution of individual length of poly(U) in each fraction. For each lane in panel A, signal from individual poly(U) length \((U_i, i = 2 \ldots 36)\) was divided by the total signal from all bands \((\Sigma(U_2 \ldots U_{36})\). The data were imported into Mathematica software to generate the contour plot. The contour lines are 1, 2.8 (average if each length is equally represented), 4, 6, 10%. The number of Ded1p molecules bound is marked above the plot and illustrated in schematics to the right of the plot. Dashed lines indicate gradient fractions plotted in panel D.

(D) Distribution of individual length of poly(U) in representative gradient fractions. Normalized signals from gradient fractions corresponding to free RNA (fraction 8, blue circles), 1 Ded1p molecule bound (fraction 18, magenta circles), 2 Ded1p molecules bound (fraction 29, green circles), 3 Ded1p molecules bound (fraction 36, red circles), and 4 Ded1p molecules bound (fraction 39, yellow circles) as shown in panel C were plotted against RNA length. Dashed lines highlight length cutoffs for Ded1p binding.

8.2.6 Simulation with 5 nt binding site size does not fully explain the binding pattern

To examine the possibility that in the presence of ADP-BeF₃ or ADP-AlF₄, each Ded1p molecule binds 5 nt RNA instead of the 10 nt binding site observed with ADPNP, we simulated the binding pattern assuming a binding site of 5 nt. When each molecule randomly binds to RNA without sliding, gaps smaller than the binding site form between molecules, leading to sub-saturation of binding sites on the RNA (Figure 8.11). We explored whether the potential gaps can explain results such as the presence of 3 instead of 5 molecules of Ded1p on a 25 nt single strand (Figures 8.6A, 8.9B).

When binding adjacent to one molecule is 10-fold more favorable than binding without neighboring molecules (positive cooperativity), the Ded1p-bound status of any RNA between 5-36 nt displays perfect 5 nt binding sites per molecule (Figure 8.12A). In the absence of cooperativity, the binding site appears to be 6 nt (Figure 8.12B). However, the transition from free RNA to Ded1p-bound between 4 and 5 nt would nonetheless reveal the 5 nt binding site. When binding next to one molecule is 10-fold less favorable than binding without adjacent molecules (negative cooperativity), the binding site appears even larger, approximately 7-8 nt (Figure 8.12C). Stronger negative cooperativity (100-fold less
favorable) does not result in larger apparent binding sites (Figure 8.12D). Thus, random binding with negative cooperativity does not sufficiently explain the apparent 10 nt binding site for 10-20 nt RNA (2 Ded1p molecules), and the binding of 3 molecules to 25 nt RNA. We therefore favor a model where 5 nt allows Ded1p binding in a not fully locked-on conformation when ADP-BeF₆ or ADP-AlF₄ is ambiently present; the binding site is ~10 nt whether Ded1p is in complex with ADPNP, ADP-BeF₆ or ADP-AlF₄; additional Ded1p molecules oligomerize with stably RNA bound Ded1p dependent on the ATP analog (Figure 8.13).

**Figure 8.11 Random binding of a protein to RNA without sliding generates gaps.**

All possible cases of a protein with 5 nt binding site completely bound to a 15 nt single-stranded RNA. Each square represents one nucleotide. Black squares indicate binding by the protein. The number of protein molecules bound in each case is labeled on the right.
Figure 8.12 Simulation of binding to 5-36 nt ladder by a protein with 5 nt binding site (see Appendix 4).

For each length of RNA, all possible cases of complete binding by the hypothetical protein were considered (as illustrated in Figure 8.11). Each case was weighted by a factor $p!w^{p-g-1}$, where $p$ is the number of protein molecules bound, $w$ is the cooperativity factor, $g$ is the number of gaps. $w$ is defined by binding next to a protein molecule is how many fold more favorable than binding without the neighboring molecule. See Appendix 4 for details of the simulation. The distribution of protein binding status for each length of RNA was displayed as array plots, with the color scheme indicated on the top of the figure. Representative plots were shown with $w = 10$ (A, positive cooperativity), 1 (B, no cooperativity), 0.1 and 0.01 (C and D, negative cooperativity).

8.3 Discussion

We showed that Ded1p binds on single-stranded RNAs with each molecule occupying ~10 nt. This binding site size is in line with crystal structures of the DEAD-box protein Mss116p in complex with ADPNP, ADP-BeF$_3^-$, and ADP-AlF$_4^-$. All structures showed binding to 10 nt of poly(U) (Del Campo and Lambowitz, 2009). Ded1p-ATP analog-RNA complexes in such a conformation have the analog and Mg$^{2+}$ ion stably bound, and show half-lives of several hrs or longer (Liu, 2010) (Table 8.2).
However, when ADP-BeFx or ADP-AlF₄ is present both in the binding reaction and during sucrose gradient sedimentation, the minimal length of RNA bound by Ded1p-ADP-BeFx or Ded1p-ADP-AlF₄ is 5 nt. We favor the explanation that the Ded1p monomer on 5 nt single strand does not represent a fully closed conformation, and contains solvent-exposed ADP-BeFx which can exchange with ADP-BeFx in solution. Another notable and not mutually exclusive possibility is that Ded1p binding to ~5 nt RNA requires interaction with additional Ded1p either on RNA or in solution, but the Ded1p oligomer is not stable during centrifugation. A Ded1p conformation with reversible ATP binding has been observed in single molecule FRET experiments (Liu, 2010). Interestingly, in the crystal structures of Mss116p, the C-terminal RecA-like domain along with the Mss116p-specific C-terminal extension binds 5 nt of RNA, while the N-terminal RecA domain contacts another 5 nt of RNA (Del Campo and Lambowitz, 2009). The structure suggests existence of a relatively open conformation of Ded1p-ADP-BeFx (or Ded1p-ADP-AlF₄) when 5 instead of 10 nt single strand is available. Between 10-20 nt, shorter RNAs sedimented in apparently higher molecular weight fractions than the longer ones. One or both of the Ded1p molecules bound on the shorter RNAs may be in a more open conformation than Ded1p bound on the longer RNAs. Note that binding to incomplete sites (<10 nt) was also observed with ADPnP on duplex with 15 nt overhang (Figure 8.3C) or on 15 nt poly(U) (Figure 8.4C), and on 1-9 nt poly(U) in the absence of scavenger RNA (Figure 9.1). All these cases require at least one molecule of stably RNA bound Ded1p (10 nt binding site), where the protein is locked on the ssRNA in the presence of an ATP analog.

While we could not examine Ded1p binding to duplex RNAes by sucrose gradients, results with ADP-BeFx and ADP-AlF₄ have clear implications for duplex unwinding. In
chapter 7, we demonstrated that Ded1p bound to single-stranded regions promotes loading of additional Ded1p to duplex regions. In this chapter, we see that Ded1p molecules align on single-stranded RNA, and oligomerize with more molecules dependent on the ATP hydrolysis state (Figure 8.13). The protein-protein interaction is stable in the presence of the ground state analog ADP-BeFx ($t_{1/2} > 16$ hrs with 10, 25 nt ssRNA), but appears to require continuing presence of the transition state analog ADP-AlF$_4$. These Ded1p molecules in oligomer with tightly RNA-bound molecules are able to associate with RNAs shorter than the 10 nt binding site. This may be envisioned to take place during loading of Ded1p onto the RNA duplex.

Figure 8.13 Model for Ded1p binding to ssRNA of different length in the presence of analogs for different states of ATP hydrolysis.
Cyan ovals represent Ded1p monomers. Red rectangles indicate ATP analogs. Semi-transparent red rectangles are used for unstably bound ATP analogs (according to Figures 8.6, 8.9). ssRNAs are drawn as straight black lines without considering looping by Ded1p. Minor species for Ded1p-ATP analog binding are shown in parenthesis.
9.1 Current working model for duplex unwinding by Ded1p

According to Chapters 6-8, I summarize our current understanding of the mechanism of unwinding by Ded1p. Ded1p molecules tightly bind to single-stranded RNA in the presence of ATP or its analogs (ADPNP, ADP-AlF₄, ADP-BeFₓ). Additional Ded1p molecules associate with stably RNA-bound Ded1p with or without direct contacts to RNA themselves. These associated Ded1p molecules are capable of duplex unwinding when ATP or ADP-BeFₓ is present. One or more molecules of ATP (or ADP-BeFₓ)-bound Ded1p locally open a duplex, followed by spontaneous dissociation of remaining base pairs. ATP hydrolysis by Ded1p bound on single-stranded regions and Ded1p bound on duplex regions both lead to dissociation from RNA. It is now possible to simulate the unwinding reaction based on our knowledge of the oligomeric state of Ded1p on substrates with various overhang and duplex lengths and the kinetic parameters measured.

9.2 Duplex invasion and its relationship to Ded1p conformations

Opening of the duplex may involve Ded1p binding to transiently available 1-10 nt single-stranded regions. Preliminary results found single-stranded RNAs shorter than the 10 nt monomer binding site associated with high molecular weight complexes. Sucrose gradient
experiments in Sections 8.2.2-8.2.5 all have a large excess of scavenger RNA (73 nt) added at the end of the binding reaction, and thus only display Ded1p molecules tightly bound to the RNA substrate. When Ded1p-ADPNP binding to the poly(U) ladder was analyzed without the scavenger, free RNA fractions only contain RNAs no longer than 12 nt (Figure 9.1), consistent with the ~10 nt binding site (Figure 8.2, 8.4). Surprisingly, these short RNAs were still present in higher molecular weight fractions (cf. Figures 8.4, 9.1). This observation was consistent with our model that Ded1p molecules stably bound on ssRNAs facilitate loading of additional Ded1p molecules to ssRNA regions less than 10 nt, a mechanism possibly used to capture fraying nucleotides in duplex regions. To further test this idea, it is possible to form stable complexes of Ded1p with ATP analogs and ssRNA, and then remove unbound Ded1p and ATP analogs (e.g. from biotinyl-streptavidin immobilized ssRNA, or through size exclusion chromatography) and use the complexes for binding additional Ded1p and 1-10 nt ssRNA.

It is also likely that the heterogeneous profile for sedimentation of Ded1p bound to a 16 bp duplex containing 25 nt overhang in the absence of the scavenger (Figure 8.1) include Ded1p molecules associated with duplex regions. The distribution of species with different molecular weights may be measured with light scattering to better define the number of Ded1p molecules on duplex regions. It is possible to probe such associations with the duplex by foot printing assays. The study can also involve the use of DNA/RNA chimeras to examine localized opening (Yang et al., 2007a).
Figure 9.1 Short ssRNAs (<10 nt) associate with Ded1p-ADPNP in the absence of scavenger. A 5'-32P-labeled poly(U) ladder (1-36 nt) was subjected to Ded1p-ADPNP binding and sucrose gradient sedimentation as described in Figure 8.4, except that no scavenger RNA (73 nt) was added after the binding reaction. Samples from the fraction number indicated were analyzed by denaturing PAGE. The number of Ded1p molecules bound is indicated below the gel. Little RNA was detected beyond fraction 25 in the absence of Ded1p (data not shown).

Results in Chapter 8 suggested that Ded1p monomers bound to 5 nt RNA do not stably bind ATP analog while Ded1p monomers bound to 10 nt RNA do. Single molecule FRET experiments revealed dissociation of ATP-bound Ded1p without ATP hydrolysis (Liu, 2010). This is consistent with a model where a monomer Ded1p reversibly binds ATP when bound to less than 10 nt RNA, and only hydrolyzes ATP when bound in a closed conformation on at least 10 nt of single strand. Available crystal structures of DEAD-box proteins in complex with single-stranded RNA and ATP analog all show the closed conformation poised for ATP hydrolysis (Sengoku et al., 2006; Bono et al., 2006; Andersen et al., 2006; Del Campo and Lambowitz, 2009). Such a conformation, however, is not
compatible with a duplex structure. Consistently, Ded1p units on duplex regions show higher $K_I$ for the ATP analog ADPNP (lower affinity) than Ded1p units on single-stranded regions (Figure 7.2), suggesting a more open ATP site for Ded1p on duplex regions. It is highly likely that the ATPase site is only aligned for catalysis when a duplex is partially opened to contain a sufficiently unpaired region (~10 nt for Ded1p).

The transition from 5 nt-bound to 10 nt-bound conformation likely represent a critical step in duplex unwinding. DNA duplexes harboring a 5 nt stretch of RNA lost the stimulation by single-stranded overhang (Yang et al., 2007a). As mentioned in Section 7.3, the current data do not distinguish between the function of overhang before (loading model) and during duplex unwinding. While the loading units of Ded1p appear functional on DNA overhangs (Yang and Jankowsky, 2006; Yang et al., 2007a), the unwinding unit(s) of Ded1p may require 5-10 nt RNA in the duplex to be facilitated by the loading units (Yang et al., 2007a). Direct tests of this model require physical measurements (e.g. optical tweezers) to determine the number of base pairs opened during ATP-dependent invasion of duplex by Ded1p, thermodynamic properties of the reaction with different substrates, etc. The precipitous decrease in unwinding rate constants with increasing duplex length (Rogers et al., 1999; Yang and Jankowsky, 2005; Quansheng Yang, unpublished) suggests the same number of base pairs opened for all duplexes, followed by spontaneous dissociation of remaining base pairs. However, preliminary studies on the temperature dependence of Ded1p-catalyzed unwinding suggested the same rate-limiting step for duplexes of different length (Quansheng Yang, unpublished), more likely a step during duplex invasion than during spontaneous dissociation.
The expected conformational changes between Ded1p bound to 5 nt ssRNA and Ded1p bound to 10 nt ssRNA may be visualized by single molecule FRET with fluorophores conjugated to cysteine residues in different RecA-like domains. It is also possible to use a more direct technique such as BSS-SAXS (basis-set supported small angle X-ray solution scattering, Yang et al., 2010). BSS-SAXS fits experimentally measured X-ray scattering of protein samples to possible conformations computed from molecular dynamics simulations to determine the conformations present in the samples. This method has been used to study effects of mutations and peptide-ligand binding on solution conformations of the tyrosine kinase Hck (Yang et al., 2010).

9.3 Refinement of the unwinding mechanism with Ded1p mutants

Carefully designed mutant studies can greatly aid our understanding of the unwinding mechanism by Ded1p in molecular detail. Of particular interest is a mutation in R489 in motif VI. This arginine is believed to stabilize the transition state during ATP hydrolysis (Figure 9.2, Section 1.3.1). Fei Liu from our laboratory showed that R489A mutation diminished ATP hydrolysis and reduced unwinding activity by 100-fold (ATP or ADP-BeF₄) (Liu, 2010). Moreover, the mutant does not tightly bind RNA in the presence of ADPNP or ADP-AlF₄, but does in the presence of ADP-BeF₄. In single molecule FRET measurements with a 19 bp duplex containing a 24 nt overhang, the mutant Ded1p displayed two RNA bound states identical to wtDed1p, but with a shift in the distribution between states. It will be interesting to see whether a mutant such as R489A can still tightly bind to 10 nt RNA and recruit additional Ded1p molecules (Chapter 8), and whether its unwinding defect can be rescued by pre-loaded wtDed1p on single-stranded overhang (Chapter 7).
Figure 9.2 Binding of ATP by conserved helicase motifs.
ATP is in schematic representation. Helicase motifs involved are labeled on the brackets. Key amino acid residues from motifs I, II and VI are shown. The arginine from motif VI is R489 in Ded1p.

9.4 Ded1p loading and unwinding on physiological RNA targets

Chapters 7 and 8 of this thesis investigated substrate loading and unwinding by Ded1p with model substrates. We are now ready to exploit the knowledge to the study of physiological RNA substrates. Dr. Ulf-Peter Guenther in our laboratory has identified a number of such RNAs from *S. cerevisiae* (Ulf-Peter Guenther, unpublished data). Studies on Ded1p and its mRNA targets will also illuminate the role of Ded1p as a translation factor (Section 6.1). These RNAs have multiple single-stranded and duplex regions according to PARS (Parallel analysis of RNA structure) (Kertesz *et al.*, 2010), which can be confirmed by chemical or enzymatic probing methods. Ded1p binding to an RNA target in the presence of ATP or its analogs after different lengths of incubation time can be detected by chemical methods such as SHAPE (Selective 2'-hydroxyl acylation analyzed by primer extension) (Merino *et al.*, 2005; McGinnis *et al.*, 2009), or by crosslinking. DEAD-box proteins mostly contact RNA on the sugar-phosphate backbone (e.g. Sengoku *et al.*, 2006; Bono *et al.*, 2006;
Andersen et al., 2006) and are expected to change the reactivity of the 2’-hydroxyl. Short pieces of the RNA crosslinked to Ded1p can also be purified and analyzed in large scale by deep sequencing. According to our model, Ded1p is expected to initially appear in single-stranded regions, and subsequently emerge in duplex regions. Sequence and structural effects on the loading and unwinding kinetics can also be assessed. The study can also be complemented by single molecule FRET measurements with strategically placed dye pairs.
Chapter 10

Materials and methods

10.1 Materials

10.1.1 Plasmids

Construction of pET15b-His$_6$-MTR4, pET15b-His$_6$-Mtr4-20p (motif VI M504I mutant) and pET15b-His$_6$-Mtr4-21p (motif II DE262,263AA mutant) was described previously (Wang et al., 2008). The E947A mutation was introduced into pET15b-His$_6$-MTR4 by PCR amplification using primers:

5’CTTTGCATTCCAAGCAGCCTGTAAAGAAGC3' and
5’CTTCTTTACAGCGTGCTTGGAATGCAAAGC3’. The entire open reading frame was verified by DNA sequence analysis. The mutated plasmid was transformed into BL21-CodonPlus(DE3) RIL strain (Stratagene), which gave better expression than the BL21(DE3) pLysS strain used for wtMtr4p (data not shown).

To generate plasmids pETduet-His$_6$-AIR2-TRF4-FLAG, the entire Air2 open reading frame was amplified from genomic DNA by Pfu turbo polymerase using primers forward JA569 (5’GATGAATTCGATGAGAAAAAAATACGGCACC3’, bold letters indicate restriction site) and reverse JA564 (5’CCCAAAAGCCTGGGTTATCTCTTCTTTCCCAAGGTAAC3’) and cloned into pETDuet-1 (Novagen) after digestion of PCR product and vector with HindIII and EcoRI. This created an N-terminal His$_6$ tagged AIR2. In a similar way, the entire open reading frame of Trf4p was amplified from genomic DNA by Pfu turbo polymerase using primers forward JA593
(5’TATGGCAGATCTCATGGGGGCAAAGAGTGTT3’) and reverse JA592
(5’CCGCTCGAGCGGTTATTGTCATCGTCGTCCTTGTAGTCAAGGGTATAAGGATTATATCCAA3’, italics represent FLAG-coding region) and cloned into the plasmid containing His6-AIR2 after digestion of PCR product and vector with XhoI and BglII. The resulting plasmid B534 was identified from a single transformant of DH5-alpha and analyzed by DNA sequence analysis. B534 was used to transform BL21(DE3) E. coli for coexpression and optimized for expression of both proteins. Mutagenesis of TRF4 to create trf4-236 has been described previously (Kadaba et al., 2006).

10.1.2 Proteins

Recombinant TRAMP complex was reconstituted and purified from E. coli strain co-expressing Trf4p/Air2p and E. coli strain expressing Mtr4p. E. coli BL21(DE3) with pETDuet-His6-AIR2-TRF4-FLAG plasmid (B534), and BL21(DE3) pLysS with pET15b-His6-MTR4 plasmid (B324) were grown to an OD600 of 0.6-0.8 and induced with 0.5 mM IPTG. Cell pellets were lysed in a buffer containing 50 mM NaH2PO4 pH 7.0, 250 mM NaCl and 10% glycerol (TRAMP binding buffer). Clarified lysates from the two strains were combined in volumes containing a roughly five-fold molar excess of Mtr4p over Trf4p/Air2p, followed by incubation with cobalt-sepharose (TALON metal affinity resin, Clontech) overnight at 4°C. Affinity column purification against His6 was carried out following standard procedures for gravity column. Purification against the FLAG tag was performed with FLAG M2 affinity gel (Sigma-Aldrich) and eluted with FLAG peptide (Sigma-Aldrich) following procedures for batch purification. The FLAG peptide was removed from the TRAMP preparation using NAP-25 columns (GE Healthcare). The final buffer contains 50 mM NaH2PO4 pH 7.0, 150-250 mM NaCl and 10% glycerol. TRAMP
concentrations were determined by Coomassie staining using bovine serum albumin (BSA) as standard. Aliquots were frozen in liquid nitrogen and stored at -80°C. Recombinant TRAMP\textsuperscript{Mtr4-20p}, TRAMP\textsuperscript{Mtr4(E947A)} and Trf4p/Air2p were purified and stored using identical procedures.

Expression and purification of Mtr4p (pET15b-His\textsubscript{6}-MTR4 plasmid), Mtr4-20p (pET15b-His\textsubscript{6}-Mtr4-20p plasmid), Mtr4p\textsuperscript{DE262,263AA} (pET15b-His\textsubscript{6}-Mtr4-21p plasmid) was described previously (Wang \textit{et al.}, 2008).

Ded1p, Mss116p and CYT-19 proteins were expressed and purified as previously described (Iost \textit{et al.}, 1999; Halls \textit{et al.}, 2007).

\textit{10.1.3 RNAs}

\textit{S. cerevisiae} tRNA\textsubscript{i}Met was transcribed \textit{in vitro} using T7 RNA polymerase (Senger \textit{et al.}, 1992) and purified with denaturing PAGE.

RNA oligonucleotides were purchased from Dharmaco. Sequences of the oligonucleotides and corresponding substrate designs are shown in Table 10.1. Radiolabeled duplex substrates were prepared as described (Jankowsky \textit{et al.}, 2000).

\textit{10.1.4 Miscellaneous reagents}

ADPNP, ADP and phosphoenolpyruvate were purchased from Sigma. Pyruvate kinase, NADH and lactate dehydrogenase were purchased from EMD biosciences. BeF\textsubscript{2}, AlF\textsubscript{3}, and NaF were purchased from Acros Organics.

Ovalbumin was purchased from Pharmacia. Aldolase from rabbit muscle was purchased from Sigma. Catalase from bovine liver was purchased from Calbiochem.

\textbf{Table 10.1 List of RNA oligos.}
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10.2 Methods

10.2.1 Polyadenylation reactions

Polyadenylation reactions were performed at 30°C in a temperature-controlled heating block in a buffer containing 40 mM MOPS pH 6.5, 100 mM NaCl, 0.5 mM MgCl₂, 5% glycerol, 0.01% Nonidet P-40, 2 mM DTT, 0.7 unit/μl Protector RNase Inhibitor (Roche). Prior to the reaction, radiolabeled RNA (0.5 nM final concentration) was incubated for 5 min with the indicated concentration of TRAMP. Reactions were started by addition of equimolar ATP and MgCl₂ at the concentrations indicated. Aliquots were removed at times indicated, and the reaction was stopped by addition of an equal volume of 80% formamide and dye markers. Samples were applied to denaturing PAGE and run to single-nucleotide resolution. Gels were dried, individual bands were visualized on a Storm PhosphorImager (GE Healthcare) and quantified using the ImageQuant 5.2 software (GE Healthcare, formerly Molecular Dynamics). For calculation of individual adenylation rate constants from the data, see Appendix 2.

10.2.2 Measurement of poly(A) tail length of hypomethylated pre-tRNAᵢMet in vivo.

Total RNA was prepared from the trm6-504 strain, which accumulates hypomethylated m¹A58 pre-tRNAᵢMet (Kadaba 2004). RNA isolation was performed according to Kohrer and Domdey (Kohrer 1991). G:I tails were appended with poly(U) polymerase (NEB). Tailing reactions were conducted with 40 pM of the in vitro pre-tRNAᵢMet mimic or 20 ng/μl total RNA in 1xMMLV-reverse transcriptase buffer (NEB, 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, pH 8.3), with 1 mM equimolar GTP and ITP, and 1 or 2 unit poly(U) polymerase (NEB) for 1 min at 37°C. Reactions were
stopped by adding 2 mM EDTA along with reverse transcription primer (5’CATACGAGCTCTTCCGATCTCCCCCCCCCCTT3’, 2-10 μM final conc.), followed by incubation at 80°C for 5 min.

First strand synthesis was performed with MMLV-RT (NEB). Reaction mixes were supplemented with 0.5 mM dNTPs, 3.3 mM MgCl₂, 1.3 units/μl murine RNase inhibitor (NEB) and 6.6 units/μl MMLV-RT (NEB) in 1x MMLV-RT buffer, and reactions were performed at 42°C for 1 hr in a final volume of 15 μl. Reactions were terminated by incubation at 95°C for 1 min. Subsequently, 0.3 μl of cDNA were amplified in a final volume of 20 μl with 1.25 unit Taq polymerase (Roche) and primers:

5’AAAACAAAAACAAAAAAGACGTGGAAGCGCGCAGGAG3’,
5’CAGGAAACAGCTATGACCCTACGAGCTCTTCCGATCT3’, at 0.375 μM each. RT-PCR was performed under standard buffer conditions with the following program: 5 min at 95°C, 20 cycles of [20 s at 95°C, 20 s at 57°C, 15 s at 72°C], 10 min at 72°C. PCR products were then re-amplified with primer

5’AAAACAAAAACAAAAACGGAAGCGCGCAGGCTCAT3’ and reverse transcription primer for 14 cycles under identical PCR conditions as described above. Specific amplification of 3’ G-tailed pre-tRNA⁺Met was verified by Sanger sequencing (data not shown).

To account for the heterogeneity of pre-tRNA⁺Met 3’-ends, the RT-PCR products were digested with MseI (NEB), which removed the tRNA moiety from the 3’ G-tailed poly(A) tails of the pre-tRNA⁺Met (Figure 5.8). Products of RT-PCR (5 μl) were incubated with 5 units of MseI (NEB) in 0.6x NEBuffer 4 for 5 hr at 37°C, followed by 20 min inactivation at 65°C, in a reaction volume of 10 μl. A dsDNA linker was then ligated to the separated poly(A)
tails. 6 μl of the restriction fragments were ligated with 0.3 μM dsDNA linker
(5’TAATACGACTCAGATATAGGAATGATACTACGGCAACCACCGAGATCTACACTCTT
TCCCTACACGGAGCTCTTCCGATCT3’) containing a 3’ TA overhang in 1X QuickLigase
buffer (66 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP, 7.5%
polyethylene glycol, pH 7.6) and 1 unit T4 DNA ligase (Roche) for 30 min at room
temperature in a final volume of 15 μl.

To finally sequence the ligation products, 0.5 μl of the ligation reaction was amplified
with 1.25 unit Herculase polymerase (Stratagene) under standard buffer conditions with
universal primer pair T7 promoter (sequence introduced by the dsDNA linker) and M13
reverse (sequence introduced by the reverse transcription primer), with the following
program: 2 min at 98° C, 23 cycles of [10 s at 98°C, 20 s at 57° C, 15 s at 72° C], 10 min at
72° C. PCR products were then purified and sequenced by ACGT Inc. (Wheeling, IL) with
the primer 5’TATAGGGAATGATACTACGGCG3’.

To calibrate the method with a known RNA, we generated in vitro an RNA
mimicking the pre-tRNA\textsubscript{iMet}. In vitro transcribed and radiolabeled tRNA\textsubscript{iMet} (2 pmol)
(Section 10.1.3) was incubated with 150 nM TRAMP in polyadenylation reaction conditions
except that 2 mM UTP-MgCl₂ was used in place of ATP-MgCl₂ (Section 10.2.1). The
reaction was subjected to denaturing PAGE (15%) and the species with 3 uridylates added
was purified. The tRNA\textsubscript{iMet+U3} was incubated with 150 nM TRAMP in polyadenylation
reaction conditions. RNA in each reaction was ethanol precipitated and purified.

10.2.3 Determination of processivity for individual adenylation steps

Polyadenylation reactions were performed as described above using 0.5 nM of 24 nt
single-stranded substrate (overtra in Table 10.1) and 150 nM wtTRAMP or Trf4p/Air2p. To
prevent enzyme rebinding (Figure 3.18), 10 μM of a scavenger RNA (73 nt RNA of unrelated sequence, SXL-BOT in Table 10.1) was added at defined times after the reaction start ($t_1$). Control reactions confirmed complete prevention of TRAMP (or Trf4p/Air2p) rebinding (data not shown). After scavenger addition, aliquots were removed from the reaction at defined times. Samples were applied to a 15% denaturing polyacrylamide gel and run to single-nucleotide resolution as described above. Gels were dried, individual bands were visualized on a Storm PhosphorImager and quantified using the ImageQuant 5.2 software (GE Healthcare). Distributions of all polyadenylated species at a given time ($t_1$) before and after scavenger addition (when this distribution no longer changed, $t_2 \approx 10$ min after scavenger addition, Figure 3.20) were determined, and processivity was calculated as described in Appendix 3.

10.2.4 Unwinding reactions with Mtr4p or TRAMP

Duplex unwinding reactions were performed at 30°C in a temperature-controlled heating block in a buffer containing 40 mM MOPS pH 6.5, 100 mM NaCl, 0.5 mM MgCl$_2$, 5% glycerol, 0.01% Nonidet P-40 (v/v), 2 mM DTT, 0.7 unit/μl Protector RNase Inhibitor (Roche). Prior to the reaction, radiolabeled duplex RNA (0.5 nM final concentration) was incubated for 5 min with the indicated concentration of TRAMP. Reactions were started by addition of equimolar dATP (or ATP where indicated) and MgCl$_2$ at the concentrations indicated. After the reaction start, aliquots were removed at times indicated, and the reaction was stopped by addition of an equal volume of 1% SDS, 0.5 mM EDTA, 20% glycerol and dye markers. Samples were applied to 15% non-denaturing polyacrylamide gel, and duplex and single-stranded RNAs were separated by electrophoresis at 15 V/cm. Gels were dried,
bands were visualized on a Storm PhosphorImager (GE Healthcare) and quantified using the ImageQuant 5.2 software (GE Healthcare).

When both unwinding and annealing reactions take place, the observed first-order rate constant $k_{obs}$ and unwinding amplitude $A_{mp}^\text{unw}$ follow equations:

$$k_{obs} = k_{unw} + k_{ann} \quad \text{(Equation 10.1)}$$

$$A_{mp}^\text{unw} = \frac{k_{unw}}{k_{unw} + k_{ann}} \quad \text{(Equation 10.2)}$$

where $k_{unw}$ and $k_{ann}$ are observed first-order unwinding and annealing rate constants respectively. Detailed derivation has been published (Yang and Jankowsky, 2005).

**10.2.5 ATPase assay with thin layer chromatography**

ATPase activities of Mtr4p and TRAMP were measured under identical buffer conditions as unwinding reactions (Section 10.2.4). For pre-steady state (enzyme excess) conditions, 20 nM duplex RNA was pre-incubated with indicated concentrations of Mtr4p or TRAMP for 5 min at 30°C. Reactions were started by addition of indicated concentrations of equimolar ATP and MgCl$_2$ containing 0.2 μM [$\gamma^{32}$P]ATP. Aliquots were removed at times and spotted on a thin layer chromatography plate. Thin layer chromatography was carried out with a buffer containing 0.75 M LiCl and 1 M formic acid to separate released phosphate from ATP. Plates were dried, spots were visualized on a Storm PhosphorImager (GE Healthcare) and quantified using the ImageQuant 5.2 software (GE Healthcare). ATPase assays with multiple enzyme turnovers were performed in the same manner except that the RNA concentration (as indicated) was greater than the enzyme concentration.
10.2.6 Annealing reactions

Annealing reactions for Mtr4p and TRAMP were performed under the same buffer condition as unwinding reactions (Section 10.2.4). Duplex RNA substrates were denatured at 95°C to generate single-stranded RNAs. Denatured single strands (0.5 nM final concentration) were incubated in reaction buffer for 5 min with 2 mM equimolar dATP and MgCl₂. Note that annealing activity does not require (d)ATP (Yang and Jankowsky, 2005; Halls et al., 2007; data not shown). I included dATP in the annealing reactions in order to maintain the same conditions as the unwinding reactions. Reactions were started by addition of 400 nM Mtr4p or TRAMP. After the reaction start, aliquots were removed at times indicated, and the reaction was quenched with an equal volume of 1% SDS, 0.5 mM EDTA, 20% glycerol and dye markers. Duplex and single-stranded RNAs were separated as described for unwinding reactions (Section 10.2.4).

10.2.7 Simultaneous polyadenylation and unwinding reactions on streptavidin beads

50 fmol of R16C-bio/*R17u duplex (asterisk marks the radiolabel) was incubated with 5 μl of Streptavidin UltraLink Resin (Thermo Scientific) in unwinding reaction buffer (200 μl total) for 1 hr at 4°C. After incubation, the resin was washed twice with reaction buffer until no radiation could be detected in the wash (data not shown) and resuspended to a 50% slurry. Reaction buffer (10 μl) containing 2 mM equimolar ATP and MgCl₂ was added to the resin, followed by incubation for 5 min on a temperature-controlled heating block set at 30°C. TRAMP (300 nM final) was then added to initiate the reaction. The reaction mixture was gently shaken every 30 s to keep the resin in suspension. 10 min after reaction start, an equal volume of stop buffer (1% SDS, 50 mM EDTA) was added to terminate the reaction.
The resin was settled by centrifugation and supernatant and resin samples were mixed with 1/4 volume of 80% formamide and dye markers. Samples were applied to a 15% denaturing polyacrylamide gel and run to single-nucleotide resolution. Gels were dried, individual bands were visualized on a Storm PhosphorImager (GE Healthcare) and quantified using the ImageQuant 5.2 software (GE Healthcare).

10.2.8 Simultaneous polyadenylation and unwinding reactions in solution

0.5 nM of *R16C-ddC/*R17u duplex (asterisks mark the radiolabels) was incubated with 150 nM TRAMP in unwinding reaction buffer for 5 min on a temperature-controlled heating block set at 30°C. Reactions were initiated by adding a final concentration of 2 mM equimolar ATP and MgCl₂. After the reaction start, two aliquots were simultaneously removed at times indicated. One aliquot was mixed with an equal volume of unwinding reaction stop buffer (1% SDS, 0.5 mM EDTA, 20% glycerol and dye markers), the other aliquot mixed with an equal volume of polyadenylation reaction stop buffer (80% formamide and dye markers). Samples were applied to a 15% denaturing gel to analyze polyadenylation and a 15% semi-denaturing (1 M urea) polyacrylamide gel to analyze unwinding respectively. The presence of urea in the semi-denaturing gel did not dissociate the duplex and provided results similar to native gel (data not shown), but allowed for better resolution of adenylated species.

10.2.9 Unwinding reactions with Ded1p

Unwinding reactions with Ded1p were performed in a buffer containing 40 mM Tris·HCl (pH 8.0), 50 mM NaCl, 0.5 mM MgCl₂, 2 mM DTT, 0.8 unit /μl RNasin, 0.01% (v/v) Nonidet P-40. 0.5 nM radiolabeled RNA substrate and 600 nM Ded1p (30 μl reaction
mixture) was incubated for 5 min in a temperature-controlled aluminum block at 19°C. After the incubation time, unwinding reactions were initiated by adding an equimolar mixture of 2 mM ATP and MgCl₂. Aliquots were removed from the reaction at the time points indicated and quenched with an equal volume of stop buffer (50 mM EDTA, 1% SDS, 0.01% bromophenol blue, 0.01% xylene cyanol, 10% (v/v) glycerol). Aliquots were then applied to a 15% nondenaturing polyacrylamide gel, and duplex and single-stranded RNAs were separated at room temperature at 10 V·cm⁻¹. Gels were dried and the radiolabeled RNAs were visualized with a PhosphorImager and quantified using ImageQuant 5.2 (GE Healthcare). Unwinding rate constants were determined as described previously (Yang and Jankowsky, 2005).

10.2.10 ATPase assay coupled to NADH oxidation

ATPase activity for Ded1p was monitored by a coupled enzyme assay using pyruvate kinase and lactate dehydrogenase to link hydrolysis of ATP to oxidation of NADH. NADH oxidation (NAD⁺) was monitored by measuring the decrease in the absorbance at 340 nm (Panuska and Goldthwait, 1980). Oxidization of 1 molecule of NADH to NAD⁺ corresponds to production of 1 molecule of ADP, therefore steady-state rate of ATP hydrolysis equals to that of NADH oxidation (Jankowsky et al., 2000). Reaction mixtures (60 μl) contained 40 mM Tris·HCl (pH 8.0), 50 mM NaCl, 0.5 mM MgCl₂, 2 mM DTT, 1 unit/μl RNasin, 0.01% (v/v) Nonidet P-40, 300 μM NADH, 2 mM phosphoenolpyruvate, and 3 units/ml of pyruvate kinase, lactate dehydrogenase. To investigate inhibition of ATP hydrolysis by ADPNP or ADP, an equimolar mixture of ADPNP and MgCl₂ or ADP and MgCl₂ was added at indicated concentrations. The inhibitors were incubated together with 2 μM RNA and 400 nM Ded1p in reaction mixtures in quartz cuvettes for 60 min (ADPNP) or 5 min (ADP) at
19°C. The reactions were initiated by adding 0.5 mM equimolar ATP and MgCl₂. The slope in the plot of absorbance versus time were determined using KaleidaGraph 3.0 (Synergy), and the rate of ATP hydrolysis is obtained by dividing the slope by the molar extinction coefficient of NADH (ε_{NADH} = 6300 M⁻¹cm⁻¹).

10.2.11 Unwinding reactions with pre-bound Ded1p-RNA complexes

Reaction mixtures (30 μl) contained the same components as regular unwinding reactions (Section 10.2.9). Radiolabeled RNA (0.5 nM final concentration) was incubated with 20 μM equimolar ADPNP and MgCl₂ and indicated concentrations of Ded1p for 60 min in a temperature-controlled aluminum block at 19°C. Reactions were then performed with 2 mM ATP-Mg²⁺ and analyzed as described for regular unwinding reactions (Section 10.2.9).

10.2.12 Equilibrium RNA protein binding reactions

Ded1p-RNA binding reactions were performed in reaction mixtures (10 μl) containing 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM MgCl₂, 2 mM DTT, 1 unit/μl RNasin, 0.01% (v/v) Nonidet P-40, 0.5 nM radiolabeled RNA substrate, 800 nM Ded1p and 0.5 mM ATP analogs (plus 0.5 mM MgCl₂) for 90 min in a temperature-controlled aluminum block at 19°C. After incubation, the reactions were terminated by adding an equal volume of 50% glycerol containing 1 μM of a 73 nt single-stranded RNA of unrelated sequence (SXL-BOT in Table 10.1) followed by 1 min incubation. The single-stranded RNA sequestered excess as well as non–stably bound protein, thus leaving only protein-RNA complexes that did not dissociate within 1 min. Terminated reactions were then analyzed by non-denaturing PAGE (7%) at 4°C. Gels were dried and the radiolabeled RNAs were visualized with a PhosphorImager and quantified using ImageQuant 5.2 (GE Healthcare).
10.2.13 Dissociation kinetics of Ded1p-RNA complexes

Reaction mixtures (30 µl) containing the same components as equilibrium binding reactions (Section 10.2.12) were incubated for 90 min in a temperature-controlled aluminum block at 19°C. The protein-RNA complexes were then challenged by adding the 73 nt single-stranded RNA (SXL-BOT in Table 10.1) to a concentration of 1 µM. At the time points indicated, aliquots were removed from the reaction and mixed with an equal volume of 50% glycerol (v/v, final concentration). Samples were then analyzed by non-denaturing PAGE (7%) at 4°C. Gels were dried and the radiolabeled RNAs were visualized with a PhosphorImager and quantified using ImageQuant 5.2 (GE Healthcare). The dissociation of protein-RNA complexes over time was fitted to a first-order rate law using KaleidaGraph 3.0 (Synergy) to determine the dissociation rate constants.

10.2.14 Sucrose gradient sedimentation of Ded1p-RNA complexes

Equilibrium binding reactions (90 µl volume) were performed as described in Section 10.2.12. Reactions were terminated by adding 1 µM (final concentration) of the 73 nt single-stranded RNA. Samples were layered onto 4.6 ml of 6-40% sucrose gradients under the same buffer conditions. Where indicated, the sucrose gradients also contained ATP analogs (ADPNP, ADP-BeFₓ or ADP-AlF₄⁻) at the concentrations indicated. The gradients were centrifuged in a Beckman SW-55 Ti rotor at 42,000 rpm for 15 hrs at 4°C. Three well-characterized proteins, ovalbumin (3.6S, 45 kDa), aldolase (7.35S, 149 kDa), and catalase (11.3S, 240 kDa) were mixed under equilibrium binding buffer conditions and centrifuged in parallel with the reaction samples. Samples were then fractionated with a fractionator (ISCO) into 50 tubes (~100 µl per tube). Radiolabeled RNA substrates in each fraction were detected
by a scintillation counter (Beckman Coulter LS 6500). Proteins used as molecular weight standards were assayed using SDS-PAGE.

10.2.15 Limited alkaline hydrolysis

A 36 nt poly(U) RNA was labeled with $^{32}$P at the 5’ end and subjected to limited alkaline hydrolysis in a 6 μl reaction mixture containing 200 nM RNA, 6 M urea and 33 mM NaOH. Five identical reactions were incubated at 85°C on a temperature-controlled aluminum heat block for 2, 4, 6, 8, 10 min respectively, and quenched by addition of 200 μl 1 mM HCl in 10 mM Tris pH 8.0. To precipitate the RNA, 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol were added to the samples and incubated together on dry ice for 2 hrs. After centrifugation at 16,100 g for 30 min, the supernatant was removed and the pellet was washed with 600 μl 70% ethanol (v/v). The final pellet was resuspended in water and the concentration of radiolabeled RNA was determined with a scintillation counter (Beckman Coulter LS 6500). Poly(U) ladders produced with different duration of alkaline hydrolysis (2-10 min) were analyzed by denaturing PAGE (20%, data not shown), and then combined to generate a uniform distribution of 1-36 nt poly(U).

10.2.16 Sucrose gradient sedimentation of Ded1p bound to single-stranded RNA ladder

Equilibrium binding reactions were performed as described in Section 10.2.14 except that 2 nM of radiolabeled poly(U) ladder was used as the RNA substrate and that a final concentration of 3 μM of the 73 nt single-stranded RNA was used to terminate the reaction. Sucrose gradient sedimentation was performed as described in Section 10.2.14. Samples from the fractions indicated were resolved by denaturing PAGE (20%) to single-nucleotide
resolution. Gels were dried and the radiolabeled RNAs were visualized with a PhosphorImager and quantified using ImageQuant 5.2 software (GE Healthcare).

To analyze distribution of individual length of single-stranded RNA, data quantified from the gel were fit to one or more Gaussian distributions using Origin 8 (OriginLab). To create a contour plot for a gel, signal from each band ($U_i$, $i = 2\ldots36$ (nt)) was normalized by the total signal from its corresponding lane ($U_i / \Sigma(U_2\ldots U_{36})$, $i = 2\ldots36$). Contour plots were then generated using Mathematica 6 software (Wolfram Research).
Appendix 1

Contributions

Chapter 3 has been published as:


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Xuying Wang from the laboratory of James T. Anderson performed early purification and characterization of TRAMP and Trf4p/Air2p. Figure 3.1 shows her data. Fei Liu wrote the computer routines for the analyses of individual adenylation rate constants and processivity. Appendix 2 and 3 were adapted from her writings by Dr. Eckhard Jankowsky and I. Ulf-Peter Guenther was responsible for determining the poly(A) length of tRNA$_{\text{Met}}$ by Sanger sequencing. Figure 3.8 is his work except that I prepared the *in vitro* substrate and analyzed it by denaturing PAGE, and the laboratory of Dr. James T. Anderson provided the yeast RNA preparations. Sukanya Srinivasan carried out PCR mutagenesis and purification for TRAMP$^\text{Mtr4(E947A)}$. She has also taken over the purification of wtTRAMP from me.

Chapter 4 will be included in the following manuscript:


Xuying Wang from the laboratory of Dr. James T. Anderson performed early purification and characterization of Mtr4p and TRAMP. None of her data is included.

Chapters 7 and 8 are not yet ready for publication.
Figure 7.1 is cited from Fei Liu’s PhD thesis (Liu, 2010) and Quansheng Yang measured the unwinding rate constants (Yang and Jankowsky, 2006). Fei Liu performed the ATPase assay and the equilibrium binding assay for Figure 7.2 and I measured the unwinding rate constants. Andrea Putnam determined the ATP hydrolysis rates for Figure 7.3 and I assayed for unwinding. Fei Liu also contributed Figure 7.5.

Fei Liu performed equilibrium binding reactions for Figures 8.2, 8.3, 8.8 and I carried out the sucrose gradient experiments for Figure 8.3. Fei Liu determined the dissociation rate constants in Table 8.2.

Part of the data in Chapters 7 and 8 was included in Fei Liu’s PhD thesis (Liu, 2010).
Appendix 2

Calculation of individual adenylation rate constants

Individual adenylation rate constants at a given ATP concentration were determined by describing the polyadenylation reaction as a series of irreversible 1st order reactions:

\[ A_0 \xrightarrow{k_1} A_1 \xrightarrow{k_2} A_2 \xrightarrow{k_3} \ldots A_{n-1} \xrightarrow{k_n} A_n \ldots \]  (Scheme A1).

\( A_0 \) is the RNA substrate, \( A_1 \ldots n \) are species with 1…n adenylates, and \( k_1 \ldots n \) represent the pseudo-first-order rate constants for the individual adenylation steps. The corresponding system of differential equations is as follows:

\[
\begin{align*}
\frac{dA_0}{dt} &= -k_1A_0 \\
\frac{dA_1}{dt} &= k_1A_0 - k_2A_1 \\
\frac{dA_2}{dt} &= k_2A_1 - k_3A_2 \\
&\quad \vdots \\
\frac{dA_{n-1}}{dt} &= k_{n-1}A_{n-2} - k_nA_{n-1} \\
\frac{dA_n}{dt} &= k_nA_{n-1}(-k_{n+1}A_n)
\end{align*}
\]  (Equations A1),

Laplace transformations of both sides of these equations with the initial conditions \( A_0(0) = 1, A_1\ldots n(0) = 0 \), yield:

\[
\begin{align*}
a_0(s) &= \frac{1}{s + k_1} \\
a_i(s) &= k_i \frac{a_{i-1}(s)}{s + k_i}, \quad i = 1 \ldots n, \quad \text{(Equations A2)}.
\end{align*}
\]
Here, \(a_0(s)\) and \(a_i(s)\) are concentrations of the substrate \(A_0\) and the species \(A_i\) as functions of the Laplace variable \(s\). Laplace transformations were defined as:

\[
F(s) = L[f(t)] = \int_0^{\infty} f(t)e^{-st}dt \quad \text{(Equation A3)}
\]

with \(s\) being the Laplace variable, and \(L\) the Laplace operator.

We defined:

\[
g_k^i(s) = L[G_k^i(t)] = \frac{1}{s + k_i}, \quad i = 1\ldots n+1 \quad \text{(Equation A4)},
\]

rearranged Equations A2 according to:

\[
\begin{cases}
a_0(s) = g_{k_1}(s) \\
a_i(s) = g_{k_{i+1}}(s) \prod_{j=1}^{i} k_j g_{k_j}(s), \quad i = 1\ldots n, \quad (\text{Equations A5}),
\end{cases}
\]

and substituted \(g_{k_{i+1}}\) (Equation A4) to obtain explicit kinetic descriptions of time courses for all individual adenylated species:

\[
\begin{cases}
A_0(t) = e^{-k_1t} \\
A_i(t) = \prod_{j=1}^{i} k_j \sum_{j=1}^{i+1} \frac{e^{-k_j t}}{\prod_{p=1, p\neq j}^{i+1} (k_p - k_j)}, \quad i = 1\ldots n, \quad (\text{Equations A6}).
\end{cases}
\]

If all steps have identical rate constants \((k_i = k_u (i = 1\ldots n))\), Equations A6 adopt the form published by (Lucius \textit{et al.}, 2003).

To calculate the rate constants from the measured time courses, we implemented Equations A6 in a computer routine using the \textit{Mathematica} 6 platform (Wolfram Research). The routine imports the experimentally measured time traces for individual species, performs non-linear least-square fitting, calculates the rate constants and displays plots for time courses of individual species along with the fit (Figure 3.3).
The computer routine is shown below. Texts between asterisks in parentheses are annotations that do not affect the program. Specifications for plots (labels, plot range, point size, etc.) determine the appearance of the plots within the program, while data can be exported and re-plotted in programs such as Origin (OriginLab), KaleidaGraph (Synergy software). Values of parameters (n, j, T) and the route for the data file need to be changed for each experiment.

L="250 mM NaCl, Amicon Ultra-4";(*name of experiments*)
n=6;(*number of time points*)
n=n+1;
j=20;(*number of intermediates*)
j=j+2;
Subscript[I,amp]=0.15;(*amplitude of intermediates*)
T=10;(*duration of experiments*)
T=T+1;

(******************Reading Data From Computer**********************)
(data=Import["Documents/Research/Data analysis/TRAMP polyadenylation/TRAMP prep activity/TRpR24-10222009 /250Am.xls"];
A=data[[1]];
Print["Time="Time=A[[1]]];
For[m=2,m<j+1,m++,Subscript[b,m-2]=A[[m]];
Subscript[data,m-2]=Table[{Time[[j]],
Subscript[b,m-2][[j]},{j,2,n}]];
Print[Subscript[Data,m-2]="",Subscript[data,m-2]];

(******************Calculate Time Course**********************)
(**********LSQ Fitting**********)
For[w=1,w<j,w++,Subscript[s,0]=FindFit[Subscript[data,0],{Subscript[a,0][t]},{Subscript[k,1]},t];
Rates=Table[Subscript[s,i-1][[1]],{i,1,w}];
Subscript[s,w]=FindFit[Subscript[data,w],{Subscript[a,w][t],{Subscript[k,w+1]},t}/.Rates; 

(************************Rates for Each Step*************************) 
(************************************************************************) 
Rates 
Print[""]; 
r=Table[{i,Subscript[k,i]},{i,1,j-1}]; 
ListPlot[r/.Rates,PlotRange>{{0,j},{0,4}},PlotStyle->{PointSize[0.03],Red},AxesLabel->{Steps,rates[min^-1]}, 
Filling->Axis,FillingStyle->Green,PlotLabel->L] 

(************************Individual Fitting*************************) 
(************************************************************************) 
Subscript[F,0]=Plot[Subscript[a,0][t]/.Rates,{t,0,T}, 
PlotRange>{{0,T},{0,1}},PlotStyle->{PointSize[0.03],RGBColor[0,0,1]},AxesLabel->{time[min],Fraction}]; 
Subscript[D,0]=ListPlot[Subscript[data,0], 
PlotRange>{{0,T},{0,1}},PlotStyle->{PointSize[0.03],RGBColor[0,0,1]},AxesLabel->{time[min],Fraction}]; 
Subscript[graph,0]=Show[{Subscript[F,0],Subscript[D,0]}, 
PlotRange>{{0,T},{0,1}},PlotLabel->"PolyA (+0Red:Real)"]; 
Print[Subscript[graph,0]]; 
Print[""]; 
For[p=1,p<j-1,p++,h=RandomReal[{0,0.9}];l=RandomReal[{0,0.9}]; 
d=RandomReal[{0,0.9}]; 
Subscript[F,p]=Plot[Subscript[a,p][t]/.Rates,{t,0,T}, 
PlotRange>{{0,T},{0,Subscript[I,amp]}},PlotStyle->{PointSize[0.03],RGBColor[h,l,d]}, 
AxesLabel->{time[min],Fraction}]; 
Subscript[D,p]=ListPlot[Subscript[data,p],PlotStyle-> 
{PointSize[0.03],RGBColor[h,l,d]},AxesLabel->{time[min],Fraction}]; 
Subscript[graph,p]=Show[{Subscript[F,p],Subscript[D,p]}, 
PlotRange>{{0,T},{0,Subscript[I,amp]}}, 
PlotLabel->"PolyA+" p "(Red:Real)"]; 
Print[Subscript[graph,p]]; 
Print[""]; 

(*******************Overall Fitting*******************) 
(************************************************************************) 
Show[Table[Subscript[graph,i],{i,0,j-2}],PlotLabel->"PolyA"] 

Table A1  Sample input data for determination of individual adenylation rate constants (Microsoft Excel .xls format).  

<table>
<thead>
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<th>Poly(A) (nt)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
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<td>0.06744</td>
<td>0.01482</td>
<td>0.01770</td>
<td>0.01247</td>
<td>0.01740</td>
<td></td>
</tr>
<tr>
<td>1</td>
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Appendix 3

Calculation of processivity for individual reaction steps

The calculation of processivities for individual steps from experimentally determined distributions of polyadenylated RNA species (Figure 3.18) was based on the following reaction scheme that described each step as a parallel reaction of two first-order processes, adenylation \( k_{f}^{i} \) and dissociation \( k_{diss}^{i} \):

\[
\begin{align*}
T \cdot A_{0} & \xrightarrow{k_{f}^{1}} T \cdot A_{1} \xrightarrow{k_{f}^{2}} T \cdot A_{2} \xrightarrow{k_{f}^{3}} \ldots T \cdot A_{n-1} \xrightarrow{k_{f}^{n}} T \cdot A_{n} \\
\downarrow k_{diss}^{1} & \quad \downarrow k_{diss}^{2} \quad \downarrow k_{diss}^{3} \quad \downarrow k_{diss}^{n} \quad \text{(Scheme A2),}
\end{align*}
\]

\( T \) represents TRAMP or Trf4p/Air2p, \( A_{0} \) to \( A_{n} \) represent the polyadenylated species and \( T \cdot A_{n} \) marks TRAMP bound to the polyadenylated RNA.

The processivity at each step, \( P_{i} \), is defined as (Ali and Lohman, 1997):

\[
P_{i} = \frac{k_{f}^{i}}{k_{f}^{i} + k_{diss}^{i}} \quad i = 1 \ldots n, \quad \text{(Equation A7)}.
\]

The concentrations of RNA substrate and intermediates before scavenger addition, \( A_{0 \ldots n}(t_{1}) \), and those after complete dissociation of TRAMP following the scavenger addition, \( A_{0 \ldots n}(t_{1} + t_{2}) \), are related to each other according to:

\[
\begin{align*}
&A_{0}(t_{1} + t_{2}) = A_{0}(t_{1})(1 - P_{1}) \\
&A_{i}(t_{1} + t_{2}) = (A_{i}(t_{1}) + \sum_{j=0}^{i-1}(A_{j}(t_{1}) \prod_{p=j+1}^{i} P_{p}))(1 - P_{i+1}) \quad i = 1 \ldots n, \quad \text{(Equations A8)}
\end{align*}
\]
Rearranging Equations A8 yields expressions for the processivity of individual adenylation steps:

\[
\begin{align*}
P_1 &= 1 - \frac{A_0(t_1 + t_2)}{A_0(t_1)} \\
P_{i+1} &= 1 - \frac{A_i(t_1 + t_2)}{A_i(t_1)} \sum_{j=0}^{i-1} \left( A_j(t_1) \prod_{p=j+1}^{i} P_p \right) \quad i = 1 \ldots n, \quad \text{(Equations A9)}.
\end{align*}
\]

To calculate processivities from the measured distributions, Equations A9 were implemented into a computer routine using the Mathematica 6 platform.

The computer routine is shown below. Texts between asterisks in parentheses are annotations and optional trouble-shooting commands that do not affect the program.

 Specifications for plots (labels, plot range, point size, etc.) determine the appearance of the plots within the program, while data can be exported and re-plotted in programs such as Origin (OriginLab), KaleidaGraph (Synergy software). Values of parameters (n, j) and the route for the data file need to be changed for each experiment.

\[
\begin{align*}
L &= "R8S10"; \quad (* \text{name of experiments}*) \\
n &= 2; \quad (* \text{number of time points}*) \\
n &= n+1; \\
j &= 20; \quad (* \text{number of intermediates}*) \\
j &= j+1;
\end{align*}
\]

(***************Reading Data From Computer***************)

************************************

data = Import["Desktop/Mathematica excel/R8S10.xls"]; 
A = data[[1]]; 
Print["Time=", Time = A[[1]]];

For[m = 2, m < j + 1, m++, 
Subscript[b, m - 2] = A[[m]]; 
Subscript[data, m - 2] = Table[{Time[[j]], 
Subscript[b, m - 2][[j]], {j, 2, n}}; 
Subscript[b, m - 2] = Subscript[data, m - 2][[1]][[2]]]
(*Print[Subscript[Data,m-2],"="\,Subscript[data,m-2]]
Print[Subscript[B,m-2],"="\,Subscript[b,m-2]]*)

(********Possessivity for individual steps**********)
(***************************************************************************)
Subscript[AA,0]=Subscript[data,0][[1]][[2]];
Subscript[p,1]=1-Subscript[data,0][[2]][[2]]/Subscript[AA,0];
(*Print[Subscript[P,1],"="\,Subscript[p,1]]*)

For[m=2,m<j-1,m++,
Subscript[AA,m-1]=Subscript[data,m-1][[1]][[2]] +Subscript[AA,m-2]
Subscript[p,m-1];
Subscript[p,m]=1-(Subscript[data,m-1][[2]][[2]]/Subscript[AA,m-1]);
(*Print[Subscript[P,m],"="\,Subscript[p,m]]*)
Processivity=Table[Subscript[p,m],{m,1,j-2}];

Print["Processivity="\,Processivity]
ListPlot[Processivity,PlotRange->{0,j-1},{0,1}],PlotStyle->
{PointSize[0.03],Red},AxesLabel->{Steps,"Processivity"}, Filling->
Axis,FillingStyle->Green,PlotLabel->L]

Table A2  Sample input data for calculation of processivity (Microsoft Excel .xls format).

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Appendix 4

Simulation of random binding of Ded1p to ssRNA

In order to simulate random binding of Ded1p to ssRNA, I first wrote a computer
routine in Mathematica 6 (Wolfram Research) to enumerate all possible patterns of binding.
Input for the binding site size, n, is required, and the length of the longest RNA is set at 36 nt
(Line 4 of the program shown below). The program starts to enumerate binding patterns at
the minimum length for monomer binding (n, n = 5 nt for our case), and ends at 36 nt. For
each nucleotide position, binding is represented by “1” and non-binding is represented by
“0”. For example, all cases of binding to 6 nt RNA include:

\{1,1,1,1,1,0\},
\{0,1,1,1,1,1\}.

A graphical representation of binding to 15 nt RNA was shown in Figure 3.11.

The program then counts the number of cases for saturated (no gap larger than n)
binding of one molecule, two molecules, three molecules, etc. (p = 1, 2, 3…) and number of
gaps in each case (g).

This program is shown below.

```mathematica
Input[n] (*binding site size*);
L=n;
PnG={};

While[L<=36 (*RNA length*),
b=Table[0,\{L\}]
(*binding status, initially all nt positions unbound*);
m=Table[0,{IntegerPart[L/n]}]
(*bound positions, 0 at position i means Ded1p #i (left to right) is not bound*);
s=Table[1,{IntegerPart[L/n]+1}]
```
(*sites under investigation, in search for m*);
p=0(*# Ded1p bound*);

P={0};
B={Table[0, {L}]};
M={Table[0, {1 + IntegerPart[L/n]}]}
(*P,B,M collect results for p,b,m respectively*);

While[p>=0 && s[[1]]<=n,
If[p==0 || s[[p+1]]<=s[[p]]+2n-1,
If[p+1<=L/n && s[[p+1]]<=L-n+1,
  m[[p+1]]=s[[p+1]];
  b[[m[[p+1]];;m[[p+1]]+n-1]]=1;
p=p+1
(*#p Ded1p binds*);
  s[[p+1]]=s[[p]]+n
(*begin searching for available site for #(p+1) Ded1p*),
If[s[[p+1]]==s[[p]]+n,
  AppendTo[P,p];
  AppendTo[B,b];
  AppendTo[M,m];
  b[[m[[p]];;m[[p]]+n-1]]=0;
  m[[p]]=0;
  s[[p+1]]=1;
p=p-1;
  s[[p+1]]=s[[p+1]]+1]
(*when p has reached maximum or when search has reached the left
end, remove the last binding & search for the next possible site*),
If[s[[p+1]]==s[[p]]+n,
  AppendTo[P,p];
  AppendTo[B,b];
  AppendTo[M,m];
  b[[m[[p]];;m[[p]]+n-1]]=0;
  m[[p]]=0;
  s[[p+1]]=1;
p=p-1;
  s[[p+1]]=s[[p+1]]+1]
(*search for site for #(p+1) does not leave a gap big enough for
binding between #(p+1) and #p. End the current round of search &
continue with --p*)

Print["All results:p,{b1,...},{m1,...}" ];
P=Delete[P,1];
B=Delete[B,1];
M=Delete[M,1]
(*Delete the all zero preset*);
Print[P];
Print[B];
Print[M];

i=1; sB={0}; G={0}; nG={0};
While[i<=Length[P],
  AppendTo[sB,Split[B[[i]]]];
  If[sB[[i+1]][[1]]==Table[0, {Length[sB[[i+1]][[1]]]}],
    sB[[i+1]]=Delete[sB[[i+1]], 1]];
  If[sB[[i+1]][[-1]]==Table[0, {Length[sB[[i+1]][[-1]]]}],
    sB[[i+1]]=Delete[sB[[i+1]], 1]];
  AppendTo[G, sB];
  AppendTo[nG, Length[sB]];
  i=i+1]
sB[[i+1]]=Delete[sB[[i+1]],-1];
AppendTo[G,DeleteCases[sB[[i+1]],{1,___}]]; 
AppendTo[nG,Length[G[[i+1]]]];i=i+1;
sB=Delete[sB,1];
G=Delete[G,1];
nG=Delete[nG,1];
Print[sB];
Print[G];
Print["# gaps in each case of binding:"];
Print[nG];
AppendTo[PnG,{P,nG}];L=L+1;

With the results, a second program was used to calculate the distribution of the number of molecules bound, when binding randomly but non-simultaneously occurs for all molecules. Cooperativity or anti-cooperativity is considered as the advantage or disadvantage of binding next to a bound molecule (w > 1 or w < 1) (McGhee and von Hippel, 1974). The cooperativity factor w is defined as a w-fold preference for a case of binding pattern with one less gap. Thus, all cases enumerated by the first program can be weighed by 

\[ p! w^{p-g-1}, \]

where p! is the permutation for the number of molecules bound (p), as different orders of binding give the same pattern; g is the number of gaps.

The probability for a given length L of RNA to have p molecules bound (p <= L/n) is then calculated by dividing the weighed summation of all cases with the same p by the weighed summation of all cases.

The program is shown below. Results from the first program are pasted in as “PnG” and not displayed. The binding site, n, and the cooperativity factor, w, need to be given.

PnG={…}
... (*Output, omitted*)
n=5
5 (*Output*)
j=1;
Input[w];
LBPro=Table[Table[0,{IntegerPart[36/n]}],{36-n+1}];(*matrix for probability results, max # binding x all length*);
While[j<=Length[PnG],W=w^(PnG[[j,1]]-1-PnG[[j,2]])
(*cooperativity factor w, i.e. one less gap is w-fold favored in binding, W=w^(P-1-nG)*);
Pr=PnG[[j,1]]!*W;
PPr=Transpose[{PnG[[j,1]],Pr}];
PPrC=Transpose[Partition[Flatten[Tally[PPr,#1==#2&]],3]]
(*count identical cases, result shown in position 3 before transposition, in element list 3 after*);
Pro=Sort[Transpose[{{PPrC[[1]],PPrC[[2]]*PPrC[[3]]}/Total[PPrC[[2]]*PPrC[[3]]]}],
#1[[1]]==#2[[1]]&];(*# bound max-1, probability*);
i=1;Probability={Pro[[1]]};
While[i<Length[Pro]-1,
If[Pro[[i,1]]≠Pro[[i+1,1]],
AppendTo[Probability,Pro[[i+1]]],
Probability[[-1,2]]=Probability[[-1,2]]+Pro[[i+1,2]]];i=i+1]
(*combine same # bound*);
q=1;
While[q<Length[Probability],
LBPro[[-1,Probability[[q,1]]]]=N[Probability[[q,2]]];q=q+1];
j=j+1;
LBPro=Reverse[LBPro];
Print[LBPro];
ArrayPlot[LBPro,ColorFunction= Function[z,ColorData["Pastel"][1-2z]],Mesh= All]
Bibliography


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J. de la Cruz, D. Kressler, D. Tollervey, and P. Linder. Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3’ end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J*, 17:1128–1140 (1998).


