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Investigating a Role for the DEAD-box Protein 5 (Dbp5) in tRNA Regulation

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Investigating a Role for the DEAD-box Protein 5 (Dbp5) in tRNA Regulation

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To my Mom, Sister and friends who have been an endless source of support and love.

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Preface

Portions of Chapters 1 and 3 were previously published as a review article in:

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The articles above have been modified to meet formatting requirements of this thesis.

Abstract

Eukaryotic gene expression is compartmentalized with transcription of genetic information from DNA into messenger RNA (mRNA) occurring in the nucleus and translation of these messages into proteins occurring in the cytoplasm. These cellular processes are physically separated by the nuclear envelope, a double membrane system that prevents exchange of macromolecules between nuclear and cytoplasmic compartments. RNA and proteins are not able to passively diffuse across this barrier but rather require transport receptors to facilitate docking and transit in and out of the nucleus through Nuclear Pore Complexes (NPCs). A GTP driven process that requires the small Ras like GTPase Ran to regulate nucleocytoplasmic shuttling of cargo is traditionally thought to regulate non-coding RNA (ncRNA) localization. Meanwhile mRNA export is regulated by the heterodimeric transport receptor Mex67-Mtr2 (Tap-p15/NXF1-NXT1 in humans) and ATP via the activity of the DEAD box Protein 5 (Dbp5 in yeast, DDX19 in humans), which is not directly dependent on Ran function. The work presented here describes how Dbp5 also regulates ncRNA export, revealing a novel ATP driven ncRNA export pathway and broader functions of mRNA export factors Dbp5 and Mex67 as general RNA export adapters.

Chapter 1: Introduction

The budding yeast *Saccharomyces cerevisiae* genome contains ~6000 protein coding genes (~70% of the yeast genome) which can be transcribed into messenger RNA (mRNA) and translated into functional proteins [1]. However, nearly 25% of the yeast genome is predicted to encode ~2000 genes that instead contain information to transcribe non-coding RNA (ncRNA) [1]. These ncRNAs fall into different classes of transcripts that include but are not limited to rRNA, tRNA, small nuclear and small nucleolar RNA (snRNA/snoRNA).

To regulate the abundance of these diverse types of RNA transcripts, eukaryotes have evolved multiple strategies. First, 3 different RNA polymerases specialize in transcription of distinct classes of mRNA and ncRNAs [2, 3]. Second, nuclear processing of these transcripts (i.e. splicing, capping, cleavage, polyadenylation, chemical modification, etc…) is governed by shared principles but through mechanisms unique to mRNA or ncRNAs. Third, nuclear transcripts must also be packaged into concise ribonucleoprotein complexes (RNPs) that can pass across the nuclear envelope that separates the nucleus from cytoplasm. Nuclear RNP biogenesis is tightly governed by the composition of RNPs, how the architecture of these complexes change during nuclear export, and the biochemical properties that control nucleocytoplasmic shuttling of proteins across Nuclear Pore Complexes (NPCs).

1.1. Nuclear Pore Complexes and Nucleocytoplasmic Shuttling

The genetic material contained within the nucleus is physically separated by a double membrane composed of an outer and inner phospholipid bilayer called the Nuclear Envelope (NE). Bidirectional movement of proteins and RNA across this barrier is required for nuclear

processes (e.g. transcription and RNA biogenesis) to occur. However, the transport of macromolecules across the NE must be achieved by passing through size restricted pores formed by NPCs. NPCs are massive, highly conserved macromolecular structures composed of ~30 unique proteins repeated in an eight-fold symmetrical pattern (~400 proteins total) to form a ~30nm wide pore through which nucleocytoplasmic shuttling can occur [4-8]. Generally, it is understood that molecules greater than 40kDa and ~40nm in diameter cannot passively diffuse across NPCs without structural rearrangements and facilitated transport [7]. This facilitated transport is in part achieved by proteins that recognize nuclear localization (NLS) or nuclear export sequences (NES) in proteins [7, 9, 10]. The core structure of the NPC is maintained by proteins termed Nucleoporin (Nups), with a number of additional factors dynamically engaging and dissociating from pores.

Karyopherins (importins and exportins) are a class of soluble transport factors that can dynamically bind NES/NLS containing cargo, NPCs, as well as a small GTPase called Ran. Ran^{GTP} is understood to dissolve import complexes to release cargo into the nucleus, while Ran^{GDP} facilitates dissociation of cargo being exported to the cytoplasm [11-14]. The spatial localization of Ran GTPase activating protein (Ran GAP) in the cytoplasm and Ran guanine nucleotide exchange factor (RanGEF) in the nucleus generates a gradient of Ran nucleotide states across the NE which establishes directional movement of molecules in a regulated manner [10, 11, 13-16]. This GTP driven protein shuttling is further used for transport of numerous non-coding RNA transport pathways (e.g. pre-ribosomal RNA subunits) and contrasts the ATP driven directionality traditionally thought to regulate mRNA nucleocytoplasmic dynamics [17-21].

1.2. Nuclear mRNA biogenesis and processing

Nuclear mRNA biogenesis can broadly be categorized into steps that include transcription, capping, splicing and 3' end formation [22-32]. However, each of these steps feedback on each other to allow responses to changing cellular environments and this contributes to quality control of defective transcripts [28, 29]. The dynamic composition of mRNPs dictate subsequent stages of processing, however the precise sequential recruitment of factors and changes in architecture of the mRNP that occur are still active areas of investigation.

Transcription of mRNAs is performed by RNA Polymerase II (RNAPII) which also facilitates assembly of the nascent messenger ribonucleoprotein complex (mRNP). Phosphorylation of the RNAPII CTD recruits enzymes that facilitate addition of a 7-methylguanosine (m^7G) cap structure to the 5' end of nascent mRNAs [33, 34]. The m^7G cap then facilitates recruitment of the Cap-Binding Complex (CBC) consisting of Cbp80 and Cbp20 in yeast which functionally protect mRNAs from 5' exoribonucleases, promotes RNA export and facilitates translation [35-37]. The integrated control of mRNP biogenesis is in part mediated by the formation of the THO/TREX (TRanscription-EXport) complex [22, 24-27, 38]. Like capping enzymes, THO is recruited to transcription sites via the phosphorylation of RNAPII CTD [22]. The core THO complex is composed of Tho2, Hpr1, Mft1 and Thp2 which together recruit additional factors such as Sub2, Yra1 (UAP56 and Aly in metazoans) and SR proteins Gbp2 and Hrb1 to form TREX [22]. Understanding finer details of TREX recruitment to nascent mRNPs is complicated by multiple potential mechanisms and necessitates future systematic elucidation of co-dependencies. For example, Yra1, which has emerged as a central factor in promoting

RNA-RNA annealing and packaging nuclear mRNP into concise particles, can be independently recruited to mRNPs directly by Sub2, Pcf11 (cleavage and polyadenylation), and Dbp2 (DEAD-box protein involved in transcription termination/mRNP assembly) [22, 39, 40]. Furthermore, human TREX is thought to be recruited during splicing via a physical interaction between the Exon Junction Complex (EJC) and Aly (mammalian Yra1) [41, 42].

As a multi-subunit macromolecular complex TREX ultimately bridges early steps of nuclear mRNP biogenesis (transcription) with the late stages (export) [22, 24-27, 38, 41]. In yeast and mammalian models it has been proposed that TREX recruits the mRNA export factor Mex67-Mtr2 (NXF1-NXT1 in humans) to mRNA via Yra1 and Hpr1 [43-45]. This recruitment is aided by Serine-Arginine rich (SR) and SR-like proteins (Npl3, Gbp2, Hrb1 and Nab2 in yeast and SRSF1, SRSF3 and SRSF7 in humans) which also independently facilitate loading of Mex67-Mtr2 on mRNA [28]. Mex67-Mtr2 then facilitates transit of mRNPs through the NPC by physical interactions with Phenylalanine-Glycine rich Nucleoporins (FG Nups) that line the central cavity of the nuclear pore [46]. In addition to recruitment of Mex67-Mtr2, SR proteins function in transcription, packaging mRNPs, splicing, translation and quality control of nuclear intron containing mRNAs [27-29, 39, 47, 48].

Contrary to prior data, recent evidence suggests Mex67 functions as a mobile nucleoporin and does not engage mRNAs within the nucleoplasm [49]. Rather it is proposed that Mex67 binds mRNPs during export through NPCs. This hypothesis is supported by the fact that Mex67 is able to accomplish its essential cellular functions when tethered to the NPC [49]. However, these differing models are not necessarily mutually exclusive, and it is possible that Mex67 is indeed co-transcriptionally loaded on mRNA by TREX in the nucleoplasm and/or such

loading events occur proximal to NPCs through Gene-Gating [4, 50]. While limited evidence currently exists for such a model, Gene-Gating has been hypothesized as a mechanism that would allow priming of nascent transcripts for immediate export by positioning transcriptionally active gene loci near NPCs [50]. Future studies will likely lead to a universal model that reconciles new and old data for Mex67 loading on mRNPs and provide a more comprehensive understanding of how mRNA export is achieved.

A poly (A) signal (PAS) in most protein coding genes signals transcription termination and 3' end processing of mRNAs [51]. PAS transcription triggers recruitment of CPF (cleavage and polyadenylation factor) and CF (cleavage factor) complexes which are deposited on nascent pre-mRNAs [32, 51]. Although not essential, components of these complexes (e.g. Pcf11) are recruited and primed for cleavage and polyadenylation functions through binding to the RNAPII CTD in a phosphorylation regulated manner [22]. Upon cleavage, Pap1 (poly (A) polymerase) synthesizes the hallmark poly(A) tail of the mRNA. These tails are length controlled (~70nt in yeast and ~200nt in mammals) by binding of poly (A) binding proteins Nab2 and Pab1 which also protect the mRNA from degradation [22, 51].

Defective mRNAs generated during nuclear biogenesis are marked by the TRAMP (Trf4/Air2/Mtr4 Polyadenylation) complex for degradation by the nuclear exosome [30, 31]. Mechanisms regulating recognition of faulty mRNAs is not well understood but nuclear basket components of the NPC (Mlp1, Mlp2, Nup60) and other peripherally localized factors (Pml39, Pml1, Esc1) are required for pre-mRNA retention in the nucleus [28]. Additionally, SR Proteins Gbp2 and Hrb1 are reported to hold improperly processed intron containing mRNAs within the nucleus and physically associate with TRAMP to facilitate degradation. Gbp2 and Hrb1 are

proposed to only recruit Mex67-Mtr2 and promote export when mRNAs are correctly processed and spliced [28]. Upon proper packaging and recruitment of relevant export factors, mRNPs are structurally remodeled in an ATP dependent manner by the DEAD-box protein Dbp5 to confer directionality to mRNP export (described in greater detail in Section 1.4).

1.3 Nuclear tRNA biogenesis and processing

In addition to mRNAs, eukaryotic cells contain genetic information encoding numerous classes of ncRNAs (e.g., miRNAs, sn/snoRNAs, rRNA, tRNA, lncRNA etc.) [1]. All 3 eukaryotic RNA Polymerases are involved in transcription of ncRNA (RNAPI for precursor rRNA; RNAPII for subsets of miRNA, lncRNA and sn/snoRNA; RNAPIII for tRNA, 5S rRNA, and U6 snRNA and other small RNAs) [52, 53]. While general principles of nuclear quality control and processing are similarly required for ncRNAs, the precise mechanisms and factors controlling these steps are traditionally thought to be unique from those that regulate nuclear mRNA biogenesis. tRNAs represent one such class of ncRNA that requires numerous processing events to perform an essential cytoplasmic function in translation and protein production.

Transcription of tRNAs is performed by RNA Polymerase III (RNAPIII) which is composed of a core Pol III complex and transcription initiation factors, TFIIIB and TFIIIC [52, 54-56]. Synthesis of newly transcribed tRNAs is tightly modulated by growth conditions and environmental stress. The transcriptional repressor of RNAPIII, Maf1, is a key regulator of this process. During favorable growth conditions Maf1 is heavily phosphorylated by TORC1, TOR-dependent kinase Sch9 and the chromatin associated CK2 [54, 56]. This phosphorylation prevents physical interactions of Maf1 with RNAPIII and promotes cytoplasmic localization by the karyopherin Msn5, which recognizes phospho-Maf1 as cargo for nucleocytoplasmic

shuttling. Under repressive stress conditions (e.g., glucose starvation), Maf1 is dephosphorylated by protein phosphatase 4 (PP4) [55]. This allows Maf1 accumulation in the nucleus, and together with coincident CK2 dissociation from tRNA gene loci, creates a nuclear environment where Maf1 can inhibit RNAPIII mediated transcription [54-56]. Moreover, nutrient deprivation also inhibits TORC1 and Sch9 mediated Maf1 phosphorylation, further promoting a transcriptionally repressive state [55].

Pre-tRNA molecules are transcribed with 5' leader and 3' trailer sequences in eukaryotes [54, 56-64]. These sequences must be cleaved for proper function in translation and their removal allows recruitment of relevant RNA binding proteins that progress subsequent steps of nuclear biogenesis [58]. Cleavage of the 5' leader sequence is catalyzed by the multi-subunit RNP, RNase P, within the nucleus. The RNA component of RNase P (*RPR1*) performs endonucleolytic removal of the leader sequence and is supported by the protein components of the complex (which range from 1 factor in bacteria to 9-10 proteins in eukaryotes) [60, 62]. Interestingly, a number of the protein components of the RNase P RNP in eukaryotes also co-function in other RNPs (e.g. RNase MRP, telomerase RNP), potentially indicating possible mechanisms of integrated co-regulation of multiple cellular processes [65]. Recent evidence shows that newly synthesized pre-tRNAs can be capped at the 5' end of the leader with m^7G by similar machinery regulating mRNA capping [66, 67]. Some preliminary data suggests this capping and stabilization of 5' leader sequences may contribute to an uncharacterized alternative nuclear export pathway [63]. However, it is unclear how this is precisely achieved, what protein factors are involved, and whether these capped tRNAs can be recognized by CBC proteins. The 3' trailer sequence of pre-tRNAs can be removed by both an endonucleolytic

(tRNase Z) and exonucleolytic pathway (Rex1 and Rrp6) [61, 64, 68]. While both mechanisms are thought to be viable routes for processing, tRNase Z (Trz1 in yeast) seems to be preferred for longer 3' trailers [61]. Under normal conditions, La proteins (Lhp1 in yeast) bind oligo (U) sequences at the 3' ends of pre-tRNAs, protecting them from the exonucleolytic digestion [61, 64, 68, 69]. It is predicted that the exonucleolytic pathway may serve as an alternative backup processing mechanism when tRNase Z or La protein binding is inhibited, but there may be tRNA species specific substrate preferences that are still unclear [61, 68, 70]. Addition of a terminal trinucleotide CCA sequence at the 3' terminus of end processed tRNAs is critical in all eukaryotes as it serves as the site where cognate amino acids are attached [64]. This reaction is catalyzed by the ATP (CTP): tRNA nucleotidyl-transferase (Cca1 in yeast) [71].

While there is some evidence that pre-tRNA intron splicing can occur before end maturation, it is believed splicing typically follows end processing [63, 64, 66]. Intron containing tRNAs comprise 21.5% of genetically encoded tRNA genes in yeast (6% in humans) spanning 10 isotypes [72]. Splicing of these introns is catalyzed by the tRNA splicing endonuclease complex (SEN in budding yeast and TSEN in humans) [73-76]. In humans and *Xenopus,* pre-tRNA splicing occurs in the nucleus similar to mRNA splicing [64, 77-79]. However, in budding and fission yeast, SEN is tethered to the mitochondrial membrane by mitochondrial import factor Tom70 [73-76, 78]. This necessitates export of intron containing pre-tRNAs for splicing to occur in yeast.

Primary export of intron containing pre-tRNA is mediated by the β-importin Los1 (Exportin-t in humans) [80-82] (Figure 1-1). Los1 forms a complex with end matured pre-tRNAs and the GTPase Ran in its GTP bound state [58]. Structural analysis reveals 5' leader and 3'

trailer sequences occlude Los1 binding, therefore requiring proper pre-tRNA end processing for formation of the Ran^{GTP}-Los1-tRNA complex [58]. This selectivity thus serves as an early step of pre-tRNA quality control prior to export. Another β-importin, Msn5, has also been implicated in tRNA export. Msn5 exhibits selectivity for charged amino-acylated tRNAs and thus is only able to export intron-less tRNAs or re-export mature tRNAs (secondary export) that have been transported back to the nucleus by constitutive retrograde transport following cytoplasmic splicing [82, 83] (Figure 1-1). While *in-vitro* studies have reported physical interactions between Msn5 and uncharged tRNA, *in-vivo* it is thought specificity for amino-acylated tRNAs is conferred by Tef1/2 in yeast (eEF1A in humans) [82, 84]. It is predicted that Tef1/2 specifically bind charged tRNAs and that Msn5-Ran^{GTP} forms protein-protein interactions with these factors rather than the tRNA itself. However, Tef1/2 mediated recruitment of Msn5 to tRNAs has not been formally tested. Unlike Msn5, Los1 doesn't exhibit preference for charged vs uncharged tRNA species, but there is some evidence for tRNA isotype specific substrate preference between tRNA export factors through an unknown mechanism [63, 64, 85, 86].

Additional factors have been implicated to support Los1 and Msn5 mediated tRNA export [63, 84-88]. While cytoplasmic amino-acylation is likely the preferred method of charging, nucleolar charging can occur prior to secondary export [89]. The nucleolar factors Utp8 and Utp9 of the U3 snoRNA-associated protein (Utp) complex have been implicated in collecting charged tRNAs in the nucleolus and delivering them to Los1 and Msn5 in the nucleoplasm [84, 87] (Figure 1-1). This pathway is mechanistically poorly understood, and it is unclear how this "hand-off" is achieved [84, 87]. Importantly Los1 and Msn5 are both non-essential proteins and can be deleted in combination without affecting cellular viability [90].

Given the essential nature of cytoplasmic tRNAs, identification of additional tRNA export factors has been an active area of investigation. Recent evidence, including my own published data, implicates the mRNA export factors Mex67-Mtr2 and Dbp5, as well as the β-importin Crm1, in tRNA nucleocytoplasmic dynamics [63, 85, 86, 88, 91]. Mex67 and Crm1 have been proposed to function parallel to Los1 and Msn5 to support pre-tRNA export, with Mex67 having an additional unique function to bind and export pre-tRNAs prior to end processing in a "precocious export" pathway [63, 85, 86] (Figure 1-1). However, neither Mex67 or Crm1 are thought to bind tRNA directly, and it is unknown how these proteins are directed to their cognate tRNA substrates *in-vivo*.

The importance of secondary (re)export is likely conserved in higher eukaryotes as well. Despite cytoplasmic splicing being a feature unique to yeast, retrograde transport of cytoplasmic tRNAs to the nucleus occurs in multiple models, from yeast to humans [83, 90, 92-94]. This process is thought to be supported by the β-importin Mtr10 and chaperone protein Ssa2, but other factors are speculated to exist to regulate this process [82, 83, 93, 95, 96] (Figure 1-1). While retrograde shuttling is believed to be constitutive, it is proposed to be highly regulated under conditions of stress, as mature tRNAs accumulate in the nucleus under such scenarios [59, 83, 93, 94]. However, challenges discerning whether this regulation occurs at the level of re-import or re-export of mature tRNAs has limited current mechanistic understanding of this phenomenon. Retrograde transport is thought to be required for proper modification on some tRNA families in yeast [64]. Furthermore, retrograde transport is required to repair or turnover tRNAs that have entered the cytoplasm via the precocious export pathway [59]. In the

absence of retrograde transport, these aberrant tRNAs are stabilized in the cytoplasm as non-functional species that cannot participate in translation.

The recent emergence of mRNA export factors in tRNA processing and nucleocytoplasmic dynamics necessitates a more thorough mechanistic characterization of how these factors perform their functions in distinct RNA export pathways [63, 85, 86, 88]. One factor that has been identified to participate in both mRNA and tRNA export is the DEAD-box protein Dbp5 [88]. While the ability of Dbp5 and other DEAD-box proteins to cause structural changes to RNPs has been well documented, many of the details of Dbp5 function remain enigmatic due to broad functions in many gene regulatory steps. This was a primary goal of my thesis work.

1.4 Emerging molecular functions and novel roles for the DEAD-box protein Dbp5/DDX19 in gene expression

DEAD-box proteins (DBPs) function ubiquitously throughout the process of gene expression [97, 98]. With 25 of these enzymes identified in yeast and >35 in humans, they represent the largest group amongst SFII helicases [38, 97]. Generally, DBPs are composed of two RecA-like domains, containing several critical helicase motifs (Q and motif I-VI), which are connected by a flexible linker region (Figure 1-2A). This includes the namesake Asp-Glu-Ala-Asp (D-E-A-D) motif contained within motif II. Together these motifs allow DBPs to recognize and hydrolyze ATP, bind nucleic acids, and invoke structural rearrangements on nucleic acid substrates. The highly conserved architecture of DEAD-box proteins has aided scientists in understanding the mechanistic properties governing these ATPases and the diverse processes they engage through common modes of action (refer to Figure 1-2B). This includes facilitating

formation of ribonucleoprotein (RNP) complexes by acting as a stably bound component (mode 1), using an "RNPase activity" to remodel the structure of RNPs by displacing bound proteins (mode 2), or RNA duplex unwinding (mode 3).

Two DBPs, Sub2 and Dbp5, are linked specifically to the essential process of messenger RNA (mRNA) export from the nucleus [99-101]. As a component of the TREX complex, Sub2 (UAP56 in humans) serves an important role in assembly of the nuclear export competent mRNP [38, 99]. Upon reaching the cytoplasmic fibrils of the NPC, Dbp5 (DDX19 in humans) is proposed to function in remodeling these complexes to drive the terminal stages of export [100-105]. The remodeling RNPase function of Dbp5 is thought to remove export receptors (e.g. Mex67 and Nab2) and prevent re-association of the transiting mRNP with the NPC after export, thus providing directionality to the process [103, 106].

While Sub2 functions appear limited to the nuclear compartment [38, 99, 107], Dbp5 is linked to diverse roles in the nucleoplasm and cytoplasm (e.g. transcription and translation), in addition to its essential role in mRNP export at NPCs. As such, Dbp5 localization is dynamic, and it rapidly shuttles between the nucleoplasm, NPCs, and cytoplasm [102, 108]. Recently, a nuclear export sequence (NES) found in the N-terminus of Dbp5 (denoted in Figure 1-2A) was shown to contribute to movement out of the nucleus in an Crm1 mediated pathway [88]. In contrast, mechanisms that import Dbp5 into the nucleus are still unclear; however, mutations in residues involved in nucleotide binding (motif VI), hydrolysis (DEAD motif), or co-regulator (Gle1/$InsP_6$) stimulation, alter Dbp5 nuclear shuttling [88, 102, 105]. This suggests that catalytic activity and/or RNA binding modulate nuclear localization. One possibility is that nuclear shuttling is facilitated by stable binding to RNAs that undergo retrograde transport from the

cytoplasm (e.g. tRNA, TLC1 etc.) [59, 109]. An alternative hypothesis is that Dbp5 has a (yet to be identified) nuclear localization sequence (NLS) within the arginine rich motif VI. Regardless of mechanism, nucleocytoplasmic shuttling allows Dbp5 to participate in the range of roles reported for the enzyme.

The broad cellular distribution and diversity of reported functions for Dbp5 raises questions regarding how the protein is directed to each task, and how each of these functions are mechanistically connected to Dbp5 enzymology. Below I discuss what is known about biochemical properties of the Dbp5 ATPase cycle, how regulation of the ATPase cycle may promote functions of Dbp5 in different cellular contexts, and what "work" the RNA-binding and/or catalytic cycle might accomplish in each case. This will include a discussion of how co-regulators could modulate enzymatic properties of the protein, and models that accommodate the breadth of research observations related to Dbp5 over the last three decades.

1.4.1. Features of the Dbp5 ATPase cycle with RNA

Characteristic of DBP family members, Dbp5 contains defined motifs that govern nucleotide and RNA binding (Q, I, II, IV, V, VI), ATP selectivity (Q), and hydrolysis/catalytic activity (II) that have been extensively reviewed in other publications (Figure 2A) [98, 110]. The ATP binding and hydrolysis cycle promoted by these motifs facilitate a series of conformational changes that in turn regulate RNA binding. For example, coordination of ATP by motifs Q, I, II and VI promote formation of a platform for RNA binding by bringing together RNA interaction interfaces on the two RecA-like domains (motif I, IV and V). RNA binding further promotes a "closed state" for the enzyme that stimulates ATPase activity [102, 105, 111]. Importantly, conformational changes influenced by the nucleotide state of Dbp5 (e.g. ATP vs. ADP) impact

the affinity of Dbp5 for RNA. In the presence of non-hydrolysable ATP analogs (e.g. AMP-PNP) Dbp5 is reported to have a binding affinity of ~40 nM for RNA while RNA binding is not detectable with ADP [103, 104]. This relationship highlights the high level of coordination between nucleotide state, RNA binding, and ATPase stimulation that must be regulated *in vivo* to direct functional outcomes. *In vitro* studies have further shown that inorganic phosphate (Pi) release following ATP hydrolysis acts as a slow step within the ATPase cycle of Dbp5 in the presence and absence of RNA [112]. Within the RNA-stimulated ATPase cycle, RNA binding itself is also slow [112]. These represent a potential rate-limiting step within the Dbp5 ATPase cycle that may be modulated by co-regulators *in vivo* (discussed below). Similar to $Dbp5^{ATP}$, the post-hydrolysis $Dbp5^{ADP\text{-}Pi}$ state is expected to be a high-affinity RNA-binding state, which for other DBPs is key to the cellular function of the protein [113, 114]. By stabilizing this transition state and slowing Pi release, it is feasible for DBPs to form stable assemblies on RNA (e.g. eIF4AIII as part of the exon junction complex) that can direct downstream events or binding of other protein factors (Figure 1-2B; mode 1). It is not currently known if a post-hydrolysis state is functionally important for Dbp5.

An additional layer of ATPase regulation is conferred by an auto-inhibitory N-terminal alpha-helical extension in Dbp5, which can be positioned between the two catalytic domains responsible for coordinating nucleotide binding. This serves to prevent formation of the ATP-bound closed state and lowers the basal Dbp5 ATPase activity [111, 115, 116]. Dbp5 also has a higher affinity for ADP (~0.4 mM) than ATP (~4 mM) [105, 117]. Given these facts it is not surprising that in the absence of co-regulators and RNA, Dbp5 exhibits a relatively low ATP hydrolysis rate of ~0.04-0.14 s^{-1} [104, 112, 118]. Upon RNA-binding, it is envisioned that auto-

inhibition is relieved and the closed state promoted, which leads to a maximal increase in ATPase activity of ~6-20-fold [100, 101, 104, 112, 118]. It has also been observed that the extent of this stimulation may vary with different RNA substrates, with poly(A), poly(U) or poly(C) showing robust stimulation compared to poly (G) and tRNA as weaker stimulators [100, 101]. Further work is required to establish whether these differences can be explained by altered binding conformations between Dbp5 and these RNAs, which may be of biological relevance.

Unlike helicases outside of the SFII enzymes, most DEAD-box proteins (including Dbp5) are inherently non-processive and are often only able to unwind short duplexes of RNA [98, 100, 101, 104, 119, 120]. Dbp5 exhibits the ability to unwind short RNA duplexes with low melting temperatures (Tm) in an ATP dependent manner [100, 101, 104]. Dbp5 is able to perform such functions in the absence of co-regulators when provided a 2-fold excess of short duplexes containing 5´ single-stranded overhangs [104]. Earlier reports also showed Dbp5 has the capacity to unwind duplexes containing 3´ overhangs, but only in the presence of unknown co-regulators [100, 101]. Importantly, it has yet to be shown if this “helicase” activity (Figure 1-2B; mode 3) has functional significance for Dbp5 cellular roles. Due to lack of evidenced processivity, the core cellular functions of Dbp5 are not predicted to be dependent on unwinding duplexes. Instead, the proteins’ RNA binding and ATPase cycle have been proposed to drive RNPase activity (Figure 1-2B; mode 2) to facilitate functions such as mRNP export [27, 97, 98, 110, 121-123].

1.4.2. Function and Regulation of Dbp5 at NPCs

1.4.2.1. Regulation of the Dbp5 ATPase Cycle at the NPC

Published data suggests Dbp5 promotes directional mRNA export using ATP hydrolysis to displace mRNA export receptors at NPCs [101-103, 105, 106]. The observable steady-state enrichment of Dbp5p at NPCs is facilitated by an interaction with the cytoplasmic nucleoporin Nup159 (NUP214 in humans) [101, 124, 125]. Nup159 binds the NTD of Dbp5 in a manner that occludes RNA binding and would be incompatible with a closed conformation of the enzyme [101, 124, 125]. This is consistent with *in vitro* observations that nucleotide and RNA (which promote the closed state) weaken Dbp5-Nup159 binding and that Nup159 inhibits RNA-stimulated ATPase activity [101, 111, 124, 125]. Notably, disruption of the Dbp5-Nup159 interaction *in vivo* is not lethal, but cells lacking this interaction can exhibit poor growth, temperature sensitivity, and mRNA export defects, suggesting the interaction is functionally important [105, 126]. A mutant that is an exception to these phenotypes is $Dbp5^{RR}$ (R256D and R259D), which disrupts a critical salt bridge between Dbp5-Nup159, causing Dbp5 enrichment at NPCs to be lost without negatively impacting growth or bulk mRNA export status [105]. This is reported to be the result of the $Dbp5^{RR}$ mutations causing accelerated ADP release from Dbp5, which bypasses the necessity for co-regulators at NPCs to promote nucleotide exchange. Specifically, it has been suggested that Nup159 acts as a nucleotide exchange factor (NEF) in this process [105]. This is in line with structural analyses of $Dbp5^{ADP}$-Gle1/$InsP_6$-Nup159 showing that, within the ternary complex, Dbp5 RecA-like domains are positioned in an open conformation that could allow for nucleotide release [111]. In contrast, recent results reporting nucleotide turnover at the millisecond timescales (kinetics relevant to mRNA export), suggest

that Nup159 does not act as an ADP release factor [117]. Together, these data indicate that further studies are needed to understand if ADP release is a regulated event *in vivo.* Furthermore, it highlights that while the structural details of the Dbp5-Nup159 interaction are well defined, the *in vivo* function of this interaction requires investigation.

A second cytoplasmic nucleoporin, Gle1, participates in an essential interaction with Dbp5 at NPCs and is known to activate Dbp5 ATPase activity *in vitro* [104, 111, 115, 118, 126-131]. This suggests that *in vivo* Gle1 could function to accelerate a slow step of the Dbp5 RNA-stimulated ATPase cycle (i.e. P_i release and/or RNA binding). The interaction between $Dbp5^{ADP}$-Gle1 has been elucidated by x-ray crystallography and shows that Gle1 binds both RecA-like domains of $Dbp5^{ADP}$, which is aided by the endogenous small molecule inositol hexakisphosphate ($InsP_6$) that bridges the interaction between Gle1 and the CTD of Dbp5 [104, 111, 115, 127, 128]. The binding of Gle1 positions the two RecA-like domains of Dbp5 in an open conformation that would exclude RNA binding and is incompatible with the auto-inhibited conformation of Dbp5 [111]. Moreover, Gle1 accelerates RNA release from preformed Dbp5-RNA complexes *in vitro*, even when ATP hydrolysis is inhibited by mutation of the DEAD motif ($Dbp5^{E240Q}$) [111]. This suggests Gle1 could aid the release of RNA from either an ATP or post-hydrolysis ADP-Pi state. In addition, by limiting conformational flexibility of the two RecA-like domains and blocking access of the N-terminal auto-inhibitory domain, Gle1 may also promote RNA binding upon exchange of ADP for ATP. Indeed, Gle1 has been reported to promote formation of an ATP bound Dbp5 state [104, 105]. These observations raise the possibility that Gle1/$InsP_6$ binding could promote multiple rounds of Dbp5 ATP hydrolysis by enhancing aspects of both RNA binding and release. Such a paradigm might parallel conformational regulation

observed for eIF4A that transitions between open, semi-open, and closed conformations promoted by eIF4G binding in different nucleotide, RNA, and co-regulator (eIF4B) contexts [132-134]. It is expected that future structural analyses and *in vitro* biochemistry of Dbp5 with Gle1 and RNA are key to testing such possibilities.

1.4.2.2. Potential Stepwise Interactions of Dbp5 at the NPC

While questions remain as to the functions of both Nup159 and Gle1 in regulating Dbp5 ATPase activity, published data can be used to propose a series of interactions between these nucleoporins and Dbp5 to promote RNP export. In figure 1-3, multiple pathways leading to RNP binding and release from Dbp5 are presented in a series of "states". This includes potential interactions that may be occurring to support these transitions. It is not currently known if each of these states are occupied *in vivo,* what fraction of Dbp5 follows each path, and if the states occupied by Dbp5 vary with the RNP substrate. Each of these points are important issues that must be addressed in the future to further refine models of Dbp5-mediated RNP export.

Given that the Dbp5-ADP binding affinity is ~10-fold higher than Dbp5-ATP [105, 117], it is calculated that a significant fraction of Dbp5p in the cell under physiological conditions may be ADP-bound (state 1a*)[112]. Nup159 is required for enrichment of Dbp5 at NPCs, with the Dbp5-Nup159 interaction precluding RNA binding [111, 124]. When bound to nucleotide, Dbp5 binding to Nup159 is also weakened from ~20 nM to ~0.6 ($Dbp5^{ADP}$) and ~1 uM ($Dbp5^{ATP}$) *in vitro* [101, 124, 125]. These biochemical and physiological observations motivate us to propose Nup159-bound pools of Dbp5 may be ADP-bound (state 1a), but likely lack nucleotide (favoring state 1a → 1b or state 1b* → 1b transitions). Dbp5 molecules positioned at the cytoplasmic fibrils may subsequently bind ATP (state 1→2), which promotes a closed state that is primed to

engage RNA. As a result, we envision a rapidly cycling pool of Dbp5 at NPCs composed of states 1a/1a*, 1b/1b*, and 2, which fits well with measurements of the dwell time of Dbp5 at NPCs that is estimated to be ~0.05-0.8 seconds [102, 108].

This locally concentrated pool of Dbp5 would be available to participate in mRNP export due to its proximal positioning to Gle1 and the exit site of mRNAs from the nucleus. Estimates suggest that an NPC transports an mRNP every ~2-6 seconds, with the transport event itself lasting ~0.2 seconds [135-137]. The millisecond dynamics of Dbp5 cycling at NPCs in relation to the seconds frequency of export suggests that the vast majority of Dbp5 is binding and releasing NPCs (e.g. cycling on and off NPCs between state 1 and 2), without participating in an mRNA export event. This idea is supported by phenotypic characterization of the Dbp5 NES ($Dbp5^{L12A}$) and $Dbp5^{RR}$ mutants that lack an obvious steady-state localization of Dbp5 at NPCs, yet do not impact bulk mRNA export [88, 105]. Such observations further suggest a model where Dbp5 at NPCs could be engaged in non-mRNP export activities (e.g. nuclear import or ncRNA transport, see further discussion below). We raise these observed differences between kinetic measurements of mRNP export and other observations involving Dbp5 (e.g. localization and essential role in mRNP export) to highlight important questions to be addressed.

In a scenario where Dbp5 alone engages an RNP, $Dbp5^{ATP}$ would bind the RNA (state 2→3a), possibly hydrolyze ATP, and if so, transition to the ADP-Pi post hydrolysis state (state 3b) [102, 105]. From the RNA-bound state 3a or 3b, Dbp5 may subsequently release the RNA without the aid of other factors, resulting in a return to state 1. However, given the essential nature of Dbp5-Gle1 interactions [127, 138], the ability of this interaction to accelerate Dbp5 ATPase activity *in vitro* [104, 118], and Gle1/$InsP_6$ mediated RNA release from Dbp5 [111], it is

likely that Gle1 facilitates one or more of these events during RNA export. In doing so, Gle1 could engage $Dbp5^{ATP}$ (state 2 →4a), the $Dbp5^{ATP}$-RNA complex (state 3a → 4b) or post-hydrolysis complex (state 3b →4c). Notably, Gle1 bound Dbp5 (state 4a or 5) might be well positioned (i.e. auto-inhibitory domain displaced and RecA-like domains organized in an open conformation) to promote multiple rounds of RNA binding on the same or different RNA without fully releasing Dbp5 (e.g. cycling between states 4a → 4b or undergoing a transition from state 5→4a upon exchange of ADP for ATP). Through these changes in state, the binding and release of Dbp5 from an RNP would accomplish the work needed to support RNP export.

Finally, from state 5, data suggests that Nup159 binding to Dbp5 weakens the interaction between Dbp5-Gle1 [117], which may aid in the release of Gle1 from Dbp5 (state 5 → 1a). Yet, the Dbp5-Nup159 interaction is not essential [125], suggesting that other transitions occur *in vivo* (state 5→1a* or 5→4a). A return to state 1 completes the cycle described here with Dbp5 being released from the NPC or remaining associated with Nup159.

1.4.2.3. Possible RNPase Activities that Promote Dbp5 Functions at the NPC

As a result of the cycle of RNA binding and release by Dbp5, displacement of export factors would occur from the RNP. A major question is how the enzymatic cycle of Dbp5, and/or the energy derived from this activity, is ultimately used to promote export. It has been proposed that Dbp5 activity at the pore facilitates RNPase remodeling events (Figure 1-2B; mode 2) which displace mRNA export adapters (e.g. Mex67 and Nab2) from mRNAs exiting the nucleus [103, 106]. Indeed, catalytic mutants of Dbp5 have been shown to increase cellular Mex67 levels on mRNAs in yeast, and Dbp5 has been reported to displace Nab2 from RNA *in vitro* [103, 106]. For several DEAD-box proteins, including Dbp5, structural analysis reveals that

nucleotide dependent RNA binding induces a local kink in the phosphate backbone of the RNA substrate [111, 134, 139-141]. This structural rearrangement may facilitate hydrolysis independent unwinding of duplexes or displacement of proteins (Figure 1-3; state 3a), with hydrolysis ensuring constant recycling and availability of the enzyme (i.e. Dbp5 does not remain locked on RNAs in the cytoplasm/nucleus) [110]. In other cases, hydrolysis causes DBPs to transition to a higher affinity binding state that are functionally relevant [113, 140-142], which may occur here in the context of mRNP export to alter RNP structure (Figure 1-3; state 3b). An ATPase deficient mutant of $Dbp5^{E240Q}$ is lethal [102], although this does not differentiate these two possible modes of action for Dbp5 on an mRNP. As such, it is unclear if mRNP remodeling as envisioned here (i.e. displacement of export factors from the mRNP) occurs as a result of Dbp5 binding to the RNA (state 3a or 4b), hydrolysis (state 3a → 3b or 4b → 4c), or the subsequent release of Dbp5 from the RNA (e.g. state 4c→5).

Beyond the role of hydrolysis, it also remains unclear if or how Dbp5 is directed to sites where relevant export adapters are bound to mRNA. One possibility is that this is mediated through direct protein-protein interactions with export factors. For Mex67, RNA-independent interactions with Dbp5 have been reported *in-vitro*; but for Nab2, direct binding to Dbp5 has not been detected by pull down assays [103, 143]. It is also possible that the binding of Mex67 to the FG-repeats within Nup159 ideally positions the export factor next to Dbp5, but this has not been demonstrated. Interestingly, recent work has shown that fusing Mex67 to a NPC component allows for ongoing mRNA export, suggesting that any essential interactions occurring between Dbp5 and Mex67 happen at NPCs [49]. Moreover, it is thought that there are multiple export factors per mRNP, raising the possibility that multiple remodeling events

must occur either by the same enzyme or multiple Dbp5 molecules acting in concert on a single mRNP [144]. Given the eight-fold symmetry of the NPC [145], multiple molecules of Dbp5 could act simultaneously, raising further questions as to how such events would be coordinated.

Strikingly, the *in vitro* displacement of Nab2 from mRNA occurs specifically with ADP bound Dbp5, by $Dbp5^{ADP}$ appearing to decrease affinity between Nab2 and RNA (Figure 1-2B; mode 2b)[103]. As noted, $Dbp5^{ADP}$ does not bind RNA [103], suggesting Nab2 remodeling is independent of Dbp5-RNA binding, ATP hydrolysis, and the energy derived from this cycle. As such, Dbp5 may instead function by decreasing Nab2 affinity for nucleic acid substrates through a direct protein-protein interaction [103, 104]. Indeed, RNA independent interactions between Dbp5 and Mex67 are reported to be strengthened by the presence of nucleotide (ATP) *in vitro* [143]. Such changes in the bound state, e.g. Dbp5 vs. $Dbp5^{ATP}$ vs. $Dbp5^{ADP}$, might therefore facilitate nucleotide dependent interactions between Dbp5 and Nab2. Alternatively, co-factors such as Gfd1, that are present at the pore and physically and genetically interact with both proteins may also regulate this function [146]. However, as noted previously, no such direct interaction between Dbp5 and Nab2 has been reported and the mechanism underlying this unique remodeling remains unclear. Furthermore, it is not known how Dbp5 specificity for Nab2 is conferred without causing rearrangements that displace non-export receptor components of the mRNP (e.g. Pab1 is also remodeled *in vitro* by $Dbp5^{ADP}$, but is not thought to be removed during export and is present on cytoplasmic mRNPs [103]).

While the models discussed above have centered on knowledge gained by the studies of mRNA export, individual states in this cycle (Figure 1-3) may further serve roles in export of ncRNA substrates given that Dbp5 has been implicated in the export of rRNA, the telomerase

RNA TLC1, and tRNA [88, 143, 147]. It is unclear how Dbp5 supports each of these pathways and whether it utilizes the RNPase mechanisms proposed for mRNP export. For example, Gle1 stimulation of Dbp5 ATPase activity is dispensable for rRNA export, as Gle1 and Dbp5 ATPase mutants do not exhibit the dominant-negative export defects observed for bulk mRNA with these same mutants [143]. Furthermore, Neumann et al. proposed that Nup159 mediated positioning of Dbp5, rather than catalytic activity, supports rRNA export. Because Mex67 is present on translating ribosomes, it is also postulated that Dbp5 does not displace Mex67 from rRNA during export, as was reported for mRNA. Rather a nucleotide-dependent and RNA-independent physical interaction between $Dbp5^{ATP}$ (Figure 1-3; state 2) and Mex67 is speculated to prevent "back-sliding" of the ribosomal subunit particle into the nucleus [143]. It is unknown if such a mechanism would also be possible for mRNAs or tRNAs, given that rRNAs, tRNAs, and mRNAs all share the export receptor Mex67 [18, 19, 23, 46, 86, 106, 135, 148, 149]. Our recent work has indicated that a nuclear pool of Dbp5 is involved in tRNA export, raising the possibility that Dbp5 could engage tRNAs in the nucleus and transit through NPCs bound to tRNA. If so, these Dbp5 molecules transiting the pore with RNA could occupy "state 3a/3b" acting as a stable scaffold (Figure 1-2B; mode 1) [88].

Overall, while much is known about the role of Nup159 and Gle1/$InsP_6$ in regulating Dbp5 nucleotide state, RNA binding, and critical conformational changes, the precise Dbp5-dependent mechanism(s) of mRNA and ncRNA export has yet to be fully uncovered. Specifically, a lack of knowledge about the protein composition of an exporting mRNP and stoichiometry of the individual components, as well as what "work" Dbp5 performs *in vivo* during mRNP export, leaves many open questions. Currently, reconstitution of this process is an extremely technical

challenge given the size, complexity, and membrane association of NPCs. A related issue is the speed of export in the context of the models presented. *In vitro*, maximal Dbp5 ATPase activity with RNA and Gle1/$InsP_6$ is ~1 ATP/sec, which is slow as compared to the estimated ~80 ms mRNPs spend at the cytoplasmic face of an NPC during export [104, 115, 118, 135-137]. While sub-second residence times of Dbp5 at the NPC agrees with the speed of the terminal steps of export, the comparatively slow ATP hydrolysis cycle does not [102, 108]. This discrepancy is compounded by any need for multiple remodeling events. Given the rapid dynamics of Dbp5 at each NPC, one possibility is that Dbp5 does not need to complete a full ATPase cycle to promote mRNP export. Hence, we pose the following questions for consideration. Are there unknown regulators of Dbp5 that accelerate ATPase activity to a level that matches the kinetics of mRNP export? Alternatively, is it possible that not all events of the proposed mRNP export cycle occur at NPCs? Could Dbp5 leave the NPC with an mRNP for subsequent ATP hydrolysis and mRNP remodeling in the cytoplasm? This would account for the slower hydrolysis cycle of Dbp5, as well as the dynamics of Dbp5 molecules at NPCs. Does Dbp5 target and displace specific export factors from an mRNP or does it bind RNAs indiscriminately to bias directional release into the cytoplasm? Is a general RNA binding activity how Dbp5 supports the export of both mRNA and ncRNAs? If so, is mRNP remodeling ultimately the result of competition for the mRNA by abundant cytoplasmic RNA-binding proteins and the act of translation? Such questions are central to understanding NPC-associated Dbp5 functions in gene expression and can be addressed in the future using live-cell imaging approaches, *in vitro* reconstitution strategies, and the powerful genetics and cell biology of the *Saccharomyces cerevisiae* system.

1.4.3. Nuclear Functions of Dbp5

The processes of nuclear mRNA biogenesis and export are known to be coupled by the functions of various protein complexes, including THO/TREX [25]. Early observations that Dbp5 accesses the nuclear compartment led to a general hypothesis that Dbp5 could link nuclear events to mRNA export [126, 150]. For example, immunological visualization of the very large Balbiani Ring (BR) mRNP in *Chironomus tentans* indicated a potential co-transcriptional recruitment of Dbp5 and role for the protein prior to export [150]. Similarly, genetic and physical interactions were identified between Dbp5 and early transcriptional machinery in yeast [151, 152]. Estruch et al. report multiple transcription factor IIH (TFIIH) components that either suppress or exacerbate defects caused by perturbing Dbp5. Based on genetic interactions identified in their initial study and a later follow-up, the authors proposed a role for Dbp5 shortly after formation of the pre-initiation complex and prior to elongation [151, 152]. A nuclear function for Dbp5 that bridges transcription and mRNA export is an attractive model that evokes ideas such as gene-gating as an elegant mechanism to tightly control early stages of gene expression [50]. While human DDX19 has been shown to fractionate with chromatin following UV irradiation (discussed below), it is important to note that efforts to ChIP Dbp5 in yeast have not yet been successful [151, 153]. Additionally, the Dbp5 NES mutant revealed a nucleolar pool of the protein, raising the possibility of additional interactions within the nucleolus [88].

Given data indicating a potential co-transcriptional Dbp5 recruitment to chromatin [150], it is possible that these interactions could facilitate the aforementioned assembly of a larger export competent RNP (figure 1b, mode 1), similar to how eIF4AIII anchors the exon-

junction complex (EJC) onto RNA [142]. In contrast to this hypothesis, recent evidence indicates nuclear pools of Dbp5 may not be required for bulk mRNA export, rather nuclear Dbp5 may support tRNA export [88]. Furthermore, no co-regulators have been identified that can inhibit Dbp5 RNA release in a manner observed for MAGOH and Y14 with eIF4AIII [142]. While this possibility still exists for ncRNAs, especially given the observed weak activation of Dbp5 by tRNA, current evidence that nuclear pools of Dbp5 are dispensable for mRNA export has important implications for current models [88, 100, 101]. This includes the potential that: (1) Dbp5 nuclear loading onto an mRNP is not a requirement for mRNA export, (2) Dbp5 nucleocytoplasmic shuttling is not required to support mRNA export, (3) and the essential functions of Dbp5 in mRNA export are performed at the cytoplasmic face of NPCs.

Another proposed nuclear function of Dbp5 is regarding cellular response to stress. Dbp5 has been reported to accumulate in the nucleus following ethanol stress and UV irradiation [153-155]. While ethanol broadly impacts Crm1 mediated shuttling of NES-containing proteins, Hodroj et al. report that the human homolog of Dbp5 (DDX19) re-localized to the nucleus upon UV-induced DNA damage via an ATR-dependent mechanism [153, 155]. The ATR target, CHK1 kinase, phosphorylated DDX19 at residue Serine 93 (S93) inducing nuclear re-localization. It is proposed that nuclear pools of DDX19 in this context are critical to DNA metabolism and R-loop resolution. Conservation of this role has not been confirmed in yeast; however, two N-terminal phospho-sites (S69 and S86) as well as Serine-162 have been identified to be phosphorylated in response to DNA damage through large scale yeast proteomic screens [156]. Serine-86 has also been identified as a site for post-translational modification (PTM) in two separate proteomic screens [156, 157], while the S162A mutation

was reported to be temperature sensitive in a recent alanine scanning mutagenesis study of Dbp5 [88]. However, none of these PTMs have been further validated and their regulation and functional significance remain uncharacterized. Moreover, a precise role for DDX19 in R-loop metabolism has not been elucidated. Another nuclear DBP, Dbp2 (DDX5 in humans), has also been implicated in R-loop regulation in budding yeast. Specifically, Dbp2 binds RNA at sites of R-loop formation, loss of Dbp2 leads to increased R-loops, and the protein (along with fellow DBPs Ded1 and Mss116) binds and destabilizes G-quadruplex RNA in an ATP-independent manner [158-163]. The implicated role of DDX19 in R-loop metabolism raises questions as to whether the Dbp5/DDX19 may have a similar function to Dbp2 in DNA metabolism or perhaps a more unique stress specific role on chromatin [161].

Yet, as novel nuclear functions for Dbp5/DDX19 continue to be studied, it is critical to consider the linearity of the gene expression pathway and the possibility that disruptions in RNA export caused by mutation in Dbp5 may indirectly impact other nuclear processes supporting gene expression. Future studies will need to clarify if Dbp5 directly acts on chromatin, if nuclear Dbp5 has preference for transcripts that reflect a direct role in R-loop metabolism, and what role PTMs play in regulating these nuclear functions.

1.4.4. Cytoplasmic Functions of Dbp5

Like nucleoplasmic and pore associated pools of Dbp5, cytoplasmic localization of the protein has important functions in regulating gene expression. It has been reported that Gle1/$InsP_6$ mediated Dbp5 activation is important for proper translation termination [129, 164-166]. In addition, Gle1 is reported to function in translation initiation through a physical contact with eIF3 in a mechanism that is independent of $InsP_6$ and Dbp5 [130, 131]. The role of Dbp5 in

translation termination and its relationship to nonsense mediated decay (NMD) has been recently reviewed in detail by the Krebber group [121]. Briefly, during translation termination it is proposed that Dbp5 recruits the polypeptide release factor eRF1 (Sup45 in yeast) to ribosomes already containing termination factors Rli1 and eRF3 (Sup35 in yeast) at the STOP site. Functional eRF1 seems to be important for Dbp5 recruitment to the ribosome, which may also be promoted by an RNA independent interaction with ribosome-bound Rli1 during termination. Dbp5 prevents premature termination by occupying a mutually exclusive binding site in the CTD of eRF1, which prevents an interaction with eRF3 until Dbp5 is removed. This interaction between eRF1 and eRF3 is critical for progression of termination as it promotes polypeptide and tRNA release via eRF3 mediated GTP hydrolysis. Subsequent removal of eRF3 allows eRF1 to also stimulate ATPase activity of Rli1 which promotes ribosome disassembly [164, 165]. In this way, Dbp5 is proposed to help regulate the sequential progression of the terminal steps of translation prior to ribosome recycling.

Yet, many finer details of how the ATPase cycle of Dbp5 is regulated to support this mechanism are still unclear. The observation that disrupting the Nup159-Dbp5 interaction abrogates Dbp5-eRF1 interaction, has led Beißel et al. to propose a mechanism in which Nup159 mediated recycling of $Dbp5^{ADP}$ is critical for translation termination [164]. Similarly, such mutations in Nup159 also result in translation read-through defects like those reported to occur when Dbp5 function is perturbed. The idea that nucleotide state and recycling of Dbp5 is critical for cytoplasmic functions is further supported by the fact that Dbp5-eRF1 binding is stabilized in the presence of non-hydrolysable ATP analogs. As is the case for mRNA export,

further investigation of how Nup159 may alter Dbp5 nucleotide release *in vivo* is required before further conclusions can be drawn.

Nevertheless, proper ATPase function and cycling appears to be important for proper cytoplasmic homeostasis in other manners as well. It has been observed that in temperature sensitive Dbp5 catalytic mutant *rat8-2*, mRNA export factors (including Mex67 and Pab1) accumulate in RNA Export Granules (REGs) with Dbp5 that are distinct from P-bodies [167]. However, results from a genetic screen with *rat8-2* revealed synthetic lethality with P-body components, leading to the observation that REGs can coalesce with P-bodies under conditions of heat stress. These observations may indicate that without proper enzymatic cycling of Dbp5, mRNP aggregates can form in the cytoplasm. Formation of phase-separated condensates and stress granules (SGs) have been shown to be regulated by other DEAD-box proteins *in vivo* and *in vitro* (e.g. eIF4A and Dhh1) [168, 169]. Consistent with this, recent studies in mammalian systems have shown that DDX19 overexpression can prevent formation of drug-induced SGs similar to eIF4A [169]. These links between Dbp5, REGs and translation control highlight a potential requirement for Dbp5 RNPase activity to regulate RNP function and localization downstream of mRNP export.

Each of these discoveries provides a putative cytoplasmic function of Dbp5 requiring active investigation to understand Dbp5 regulated gene expression. As these avenues of research are pursued, I expect it will be critical to consider how these functions may be related to, or unique from, mRNP processing by Dbp5 at NPCs. For example, further investigation into how Dbp5/Gle1 are recruited to translating ribosomes, the spatial regulation of these roles (e.g. at NPCs or within the cytoplasmic compartment away from the NPCs), and nature of RNP

changes induced by Dbp5 will be critical. As more Dbp5 functions are reported, this will allow important distinctions between what could be multiple distinct functions performed by Dbp5 vs. a universal function for Dbp5 in regulating RNP composition in a spatially regulated manner.

1.4.5. Thesis Focus

In this study, genetic and molecular methods are employed to characterize how Dbp5 supports tRNA export and the biochemical properties of Dbp5-tRNA regulation. I show that Dbp5 functions parallel to the canonical tRNA export factor Los1. *In vivo* co-immunoprecipitation data further shows Dbp5 is recruited to tRNA independent of Los1, Msn5 (another tRNA export factor), or Mex67 (mRNA export adaptor), which contrasts with Dbp5 recruitment to mRNA that is abolished upon loss of Mex67 function. However, as with mRNA export, overexpression of Dbp5 dominant-negative mutants indicates a functional ATPase cycle and that binding of Dbp5 to Gle1 is required to direct tRNA export. Biochemical characterization of the Dbp5 catalytic cycle demonstrates the direct interaction of Dbp5 with tRNA (or double stranded RNA) does not activate Dbp5 ATPase activity, rather tRNA acts synergistically with Gle1 to fully activate Dbp5. These data suggest a model where Dbp5 directly binds tRNA to mediate export, which is spatially regulated via Dbp5 ATPase activation at nuclear pore complexes by Gle1.

1.5 Figures

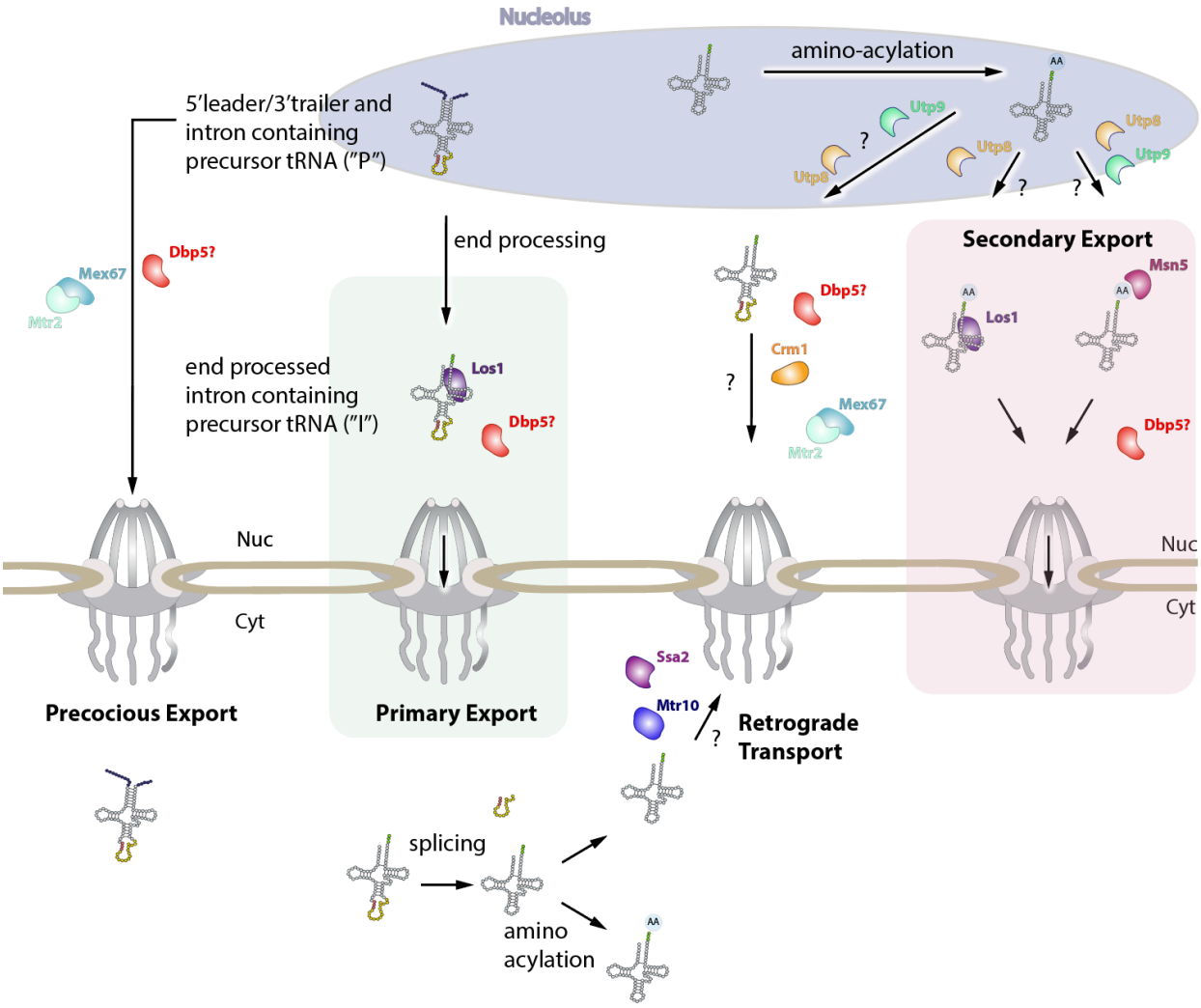
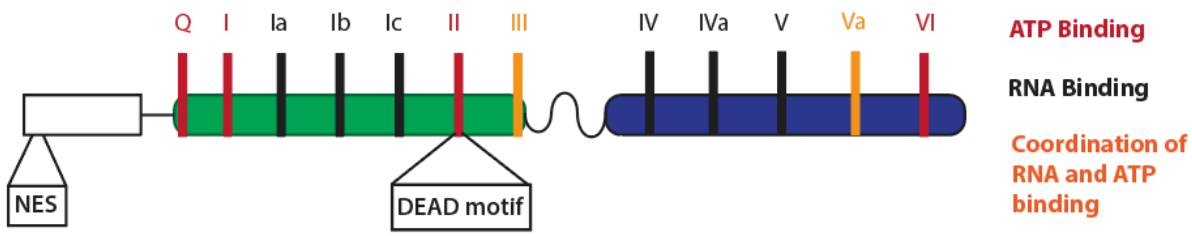

Nucleolus
amino-acylation
5'leader/3'trailer and
intron containing
precursor tRNA ("P")
Utp9
Utp8
Utp8
Utp8
Utp9
?
?
?
end processing
Dbp5?
Mex67
Mtr2
end processed
intron containing
precursor tRNA ("I")
Los1
Dbp5?
Dbp5?
Crm1
?
Mex67
Mtr2
Secondary Export
Msn5
Los1
Dbp5?
Nuc
Cyt
Nuc
Cyt
Precocious Export
Primary Export
Ssa2
Mtr10
?
Retrograde
Transport
splicing
amino
acylation

Figure 1-1. Summary of tRNA Nucleocytoplasmic Shuttling. tRNAs transport can occur via a primary, secondary, or precocious export pathway. Primary export of intron containing pre-tRNAs following end processing is mediated by Los1. Parallel to Los1, Mex67-Mtr2 and Crm1 are also believed to facilitate export of intron-containing pre-tRNAs. Upon splicing of tRNAs in the cytoplasm, tRNAs can be charged locally or undergo retrograde transport via Mtr10, Ssa2 and yet to be identified additional factors. Re-imported and intron-less tRNAs can be amino-acylated within the nucleolus. The nucleolar U3 snoRNA-associated proteins Utp8 and Utp9 collect charged tRNAs and deliver them to secondary export factors (Msn5 or Los1) for re-export. Additionally, Mex67-Mtr2 can bind and export 5' leader containing tRNAs prematurely prior to end processing through an unknown mechanism. The DEAD-box Protein Dbp5 has been previously implicated in tRNA export and could participate in any of these potential export pathways.

A.


Q
I
Ia
Ib
Ic
II
III
IV
IVa
V
Va
VI
NES
DEAD motif
ATP Binding
RNA Binding
Coordination of RNA and ATP binding

B.

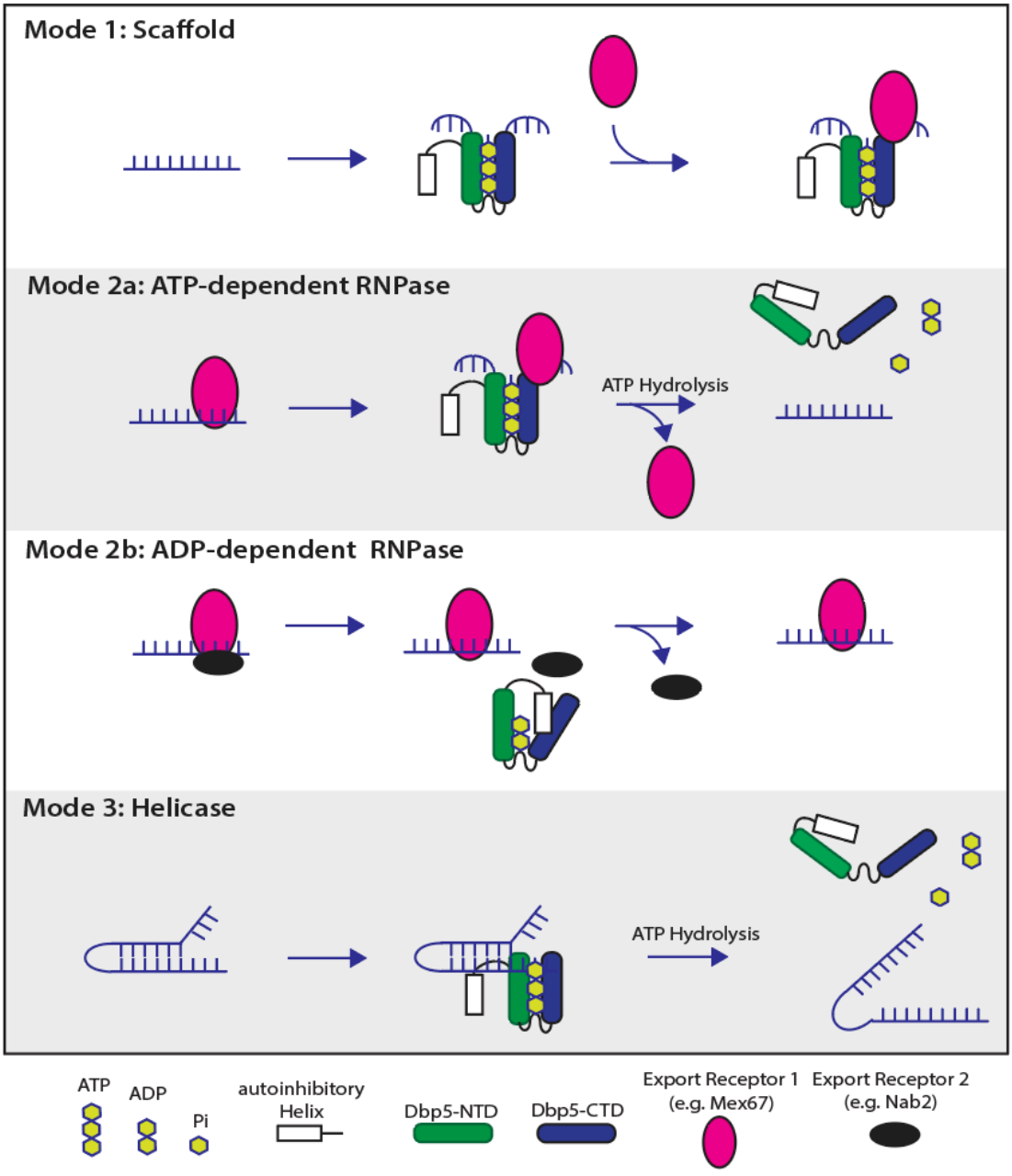

Mode 1: Scaffold
Mode 2a: ATP-dependent RNPase
ATP Hydrolysis
Mode 2b: ADP-dependent RNPase
Mode 3: Helicase
ATP Hydrolysis
ATP
ADP
Pi
autoinhibitory Helix
Dbp5-NTD
Dbp5-CTD
Export Receptor 1 (e.g. Mex67)
Export Receptor 2 (e.g. Nab2)

Figure 1-2. General organization and potential modes of action for Dbp5. (a) Diagram representation of Dbp5, including the location of the nuclear export signal (NES), RecA-like N-terminal domain (NTD), RecA-like C-terminal domains (CTD), and characteristic DEAD-box proteins motifs. Label color indicates major function associated with each motif; see figure for details. (b) Possible "modes" of action through which Dbp5 and other DBPs may engage RNA substrates. Mode 1 depicts a DBP stably binding RNA to form a scaffold on which other proteins can assemble. Mode 2 indicates an RNPase mechanism in which RNA-binding proteins (RBPs) are displaced by the DBP. Within this mode, most commonly the DBP would act in an ATP-dependent mechanism to displace RBPs through binding the RNA backbone (mode 2a). For Dbp5, data suggests that RNPase activity can result from a unique ADP-dependent mechanism that is independent of RNA-binding (mode 2b). Mode 3 depicts a "helicase" mechanism by which duplexed RNA can be disrupted through the RNA binding and ATP hydrolysis cycle of a DBP.

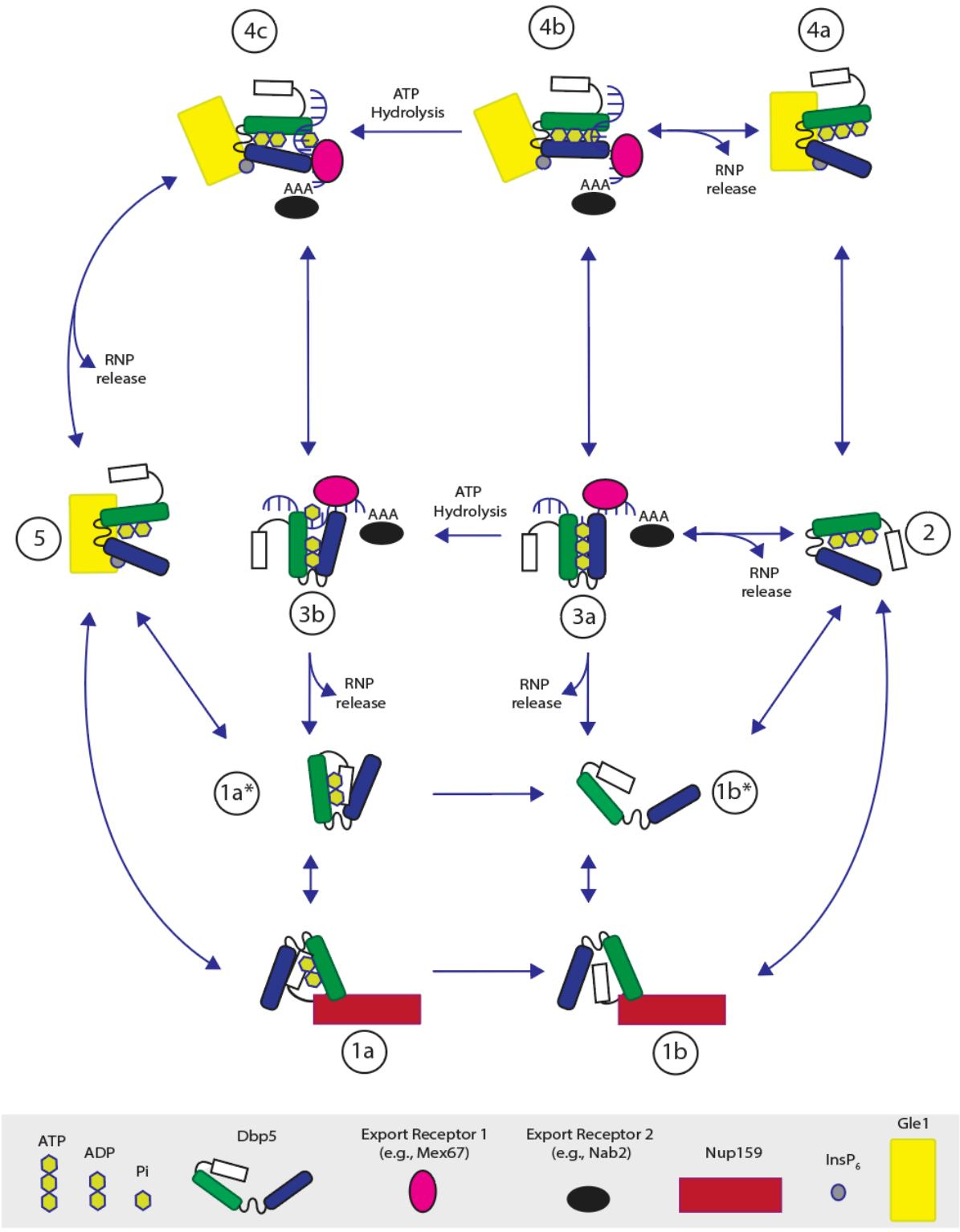

4c
4b
4a
ATP
Hydrolysis
AAA
AAA
RNP
release
RNP
release
5
AAA
ATP
Hydrolysis
AAA
2
RNP
release
3b
3a
RNP
release
RNP
release
1a*
1b*
1a
1b
ATP
ADP
Pi
Dbp5
Export Receptor 1
(e.g., Mex67)
Export Receptor 2
(e.g., Nab2)
Nup159
InsP6
Gle1

Figure 1-3. Potential binding states of Dbp5 at NPCs supporting RNP export. A stepwise cycle of possible interactions involving Dbp5, ATP, ADP, inorganic phosphate (P_i), Gle1/$InsP_6$, Nup159, RNA, and RBPs at the cytoplasmic face of an NPC is depicted. Arrowheads indicate directionality with many events having the potential to proceed in either direction. Note that not all possible states are shown due to space limitations and the position of the Dbp5 NTD in each state is postulated as current x-ray crystallography data lacks information on the position of the Dbp5 NTD domain. The cycle is presented starting with Dbp5 or $Dbp5^{ADP}$ undergoing cycles of binding (states 1a/b) and release (states 1a*/b*) from Nup159, which enriches Dbp5 at the cytoplasmic fibrils of the NPC. Nucleotide exchange allows Dbp5 to enter an ATP (state 2), which weakens interaction with Nup159. $Dbp5^{ATP}$ can either proceed to bind RNA directly (state 3a) or bind Gle1/$InsP_6$ (state 4a) prior to binding RNA (state 4b). RNA binding promotes a closed conformation and displacement of the auto-inhibitory NTD of Dbp5 (states 3a or 4b) with ATP hydrolysis leading to formation of a $Dbp5^{ADP\text{-}Pi}$ complex (state 3b or 4c). Gle1p/$InsP_6$ binding to both RecA-like domains of Dbp5 (state 4a or 5) relieves auto-inhibition of Dbp5 and promotes separation of the NTD and CTD domains to potentially promote the release of RNA from either the ATP or ADP-Pi bound forms of Dbp5 (states 4c→5 or 4b→4a). In the $Dbp5^{ATP}$-Gle1/$InsP_6$ complex (state 4a), which could be formed through the exchange of ADP for ATP from state 5, the domains of Dbp5 may be well positioned for RNA binding (state 4a→4b), which could promote cycles of RNA-binding and release without ATP hydrolysis (4a←→4b). Finally, a Nup159-Dbp5 interaction may promote release of Gle1/$InsP_6$ from Dbp5 (state 5→1a) resulting in regeneration of state 1, thus completing the cycle. Within this scheme, RNP remodeling to promote export (i.e. displacement of export factors from the RNP) may occur as a result of

Dbp5 binding to the RNA (state 3a or 4b), hydrolysis (state 3a → 3b or 4b → 4c), or the subsequent release of Dbp5 from the RNA (e.g. state 4c→5). Additionally, the $Dbp5^{ADP}$-Gle1/$InsP_6$ complex (state 5) would be a state capable of RNA binding independent remodeling (Figure 1-2B; mode 2b).

Chapter 2: Materials and Methods

Yeast strains generation and growth conditions

A list of all yeast strains used in this study is provided in Table S1. Deletion mutants and C-terminal tagging was conducted by PCR based transformations and confirmed by colony PCR. All yeast transformations were conducted using previously published standard high-efficiency LiAc, ssDNA, PEG protocol [170]. Yeast were cultured in YPD or synthetic complete (SC) media when indicated to mid log growth. For overexpression of Dbp5 ATPase mutants, untagged Dbp5 or *dbp5*^{R426Q}, *dbp5*^{R369G}, or *dbp5*^{E240Q} were integrated at URA3 locus under control of pGAL promoter in a wild-type strain with Dbp5 expressed from its endogenous locus and promoter. pGAL expression was induced by first derepressing the promoter with growth in YP media with 2% raffinose overnight followed by a shift to 2% galactose containing media for 6 hours. Overexpression was halted by addition of 2% glucose and further incubation for 1 hour.

Crosslinking and Analysis of cDNAs (CRAC)

CRAC was performed as described in Delan-Forino et al 2019 [171]. 2.5L Yeast cultures were crosslinked on the MegaTron for 100seconds (~1699mJ/cm^2). First Immunoprecipitation was performed using IgG Sepharose beads to isolate Protein -TEV-His6-A tagged Dbp5 followed by sequential washes in TN150 (50mM Tris-HCl pH7.8, 150mM NaCl, 0.1% IGEPAL, 5mM beta-mercaptoethanol), TN1000 (50mM Tris-HCl pH7.8, 1M NaCl, 0.1% IGEPAL, 5mM beta-mercaptoethanol) and TN150 respectively. Protein was eluted by TEV cleavage. Eluate was treated with RNaseIT to generate footprints and reaction was stopped by addition of 6M

Guanidinium-HCl. Dbp5-RNA complexes were then enriched on Nickel-NTA beads overnight. Resin was loaded into SnapCap columns and complexes were washed 2x in Wash Buffer I (6M Guanidinium-HCl, 50mM Tris-HCl pH7.8, 300mM NaCl, 10mM Imidizole pH8.0, 0.1% Nonidet P-40, and 5mM beta-mercaptoethanol) followed by 2x in PNK Buffer (50mM Tris-HCl pH7.8), 10mM MgCl2, 0.1% Nonidet P-40, and 5mM beta-mercaptoethanol). Dephosphorylation of RNA 3' ends was performed using Alkaline Phosphatase (TSAP) and resin was washed again with Wash Buffer I and 1x PNK Buffer. 3' Linker Ligation of miRCat-33 Linker was carried out on beads using T4 RNA Ligase I for 4 hours at 25°C and resin was again washed with Wash Buffer I and 1x PNK buffer. 5' ends of crosslinked RNA was phosphorylated with ^{32}P-ATP using polynucleotide kinase at 37°C for 40min. Complexes were again washed and 5' adapters were ligated on beads using T4 RNA Ligase I overnight at 16°C. Resin was washed 3x in Wash Buffer II (50mM Tris-HCl pH7.8, 50mM NaCl, 10mM Imidazole pH 8.0, 0.1% Nonidet P-40, and 5mM beta-mercaptoethanol). Complexes were eluted with Elution Buffer (50mM Tris-HCl pH7.8, 50mM NaCl, 150mM Imidazole pH8.0, 0.1% Nonidet P-40, and 5mM beta-mercaptoethanol) and precipitated using tricholoracetic acid and GlycoBlue coprecipitant. Complexes were resolved on 4-12% NuPAGE gradient gels and transferred to nitrocellulose. Membranes were exposed to high sensitivity X-ray film at -80°C. X-ray films were aligned to the membrane using chemiluminescent rulers and complexes were excised. RNA was recovered by proteinase K digestion, PCI extraction and Ethanol precipitation. RT PCR was performed to generate cDNA. Uniquely barcoded cDNAs (from unique 5' linkers) from each mutant intended for pairwise comparisons were pooled and libraries were amplified by pooling 5 separate PCR reactions to control for batch effects. PCR amplified libraries were separated on 3% Metaphor gels and libraries with correct footprint sizes were excised and

purified. RNA sequencing was performed on the MiniSeq platform. Analysis of CRAC data was performed using the pyCRAC software suite [172].

Helicase Assays

Fluorescent Helicase Unwinding Assays were performed as previously reported [173]. Briefly, reactions were assembled containing 100nM dsRNA, 2.5uM Dbp5, 2mM nucleotide (where appropriate), 5uM Gle1 and $InsP_6$ (where appropriate) and 0.025U/ul RNase T1 and incubated at 37°C for indicated length of time. Reactions were stopped by addition of equal volume 2X loading buffer (20% glycerol, 0.8% SDS, 10mM EDTA, 0.04% bromophenol blue) and samples separated in native 12% polyacrylamide TBE gels at 100V at 4°C. dsRNA was generated by annealing ssRNA oligos with the following sequences:

5'/56-FAM/ rArGrCrArCrCrGrUrArArArGrA-3'

5'-rUrArArArArCrArArArArCrArArArArCrArArArArCrArArArArUrCrUrUrUrArCrGrGrUrGrCrU-3'

Working stocks of ssRNA was prepared in 1X ATPase Buffer (30mM Hepes pH 7.5, 100mM NaCl, 2mM MgCl2). Annealing was performed by mixing equimolar amounts of each probe and heating to 95°C for 5min and slowly cooling to room temperature.

Chromatin Immunoprecipitation (ChIP)

ChIP experiments were performed as described in Roberts et al 2003 and Roberts et al 2006 with some modifications [174, 175]. Cells were crosslinked at 0.3% formaldehyde for 30min and quenched with 60mM Glycine for 5min. Pellets were lysed using Lysis Buffer (50mM Hepes,

140mM NaCl, 1mM EDTA, 1% Triton, 0.4mM EDTA, 0.1% Sodium Deoxycholate, 1:5000 anti-foam, 1X Protease Inhibitor Cocktail) and cleared. Sonication of cleared lysate was performed by 1 pulse at 1.5x power. 1% of the Input was reserved and immunoprecipitation of protein A tagged Dbp5 and Rpc82 was performed using IgG conjugated Dynabeads for 1 hour at 4°C followed by 3x 5min washes using wash buffer (20mM Hepes, 110mM KOAc, 2mM MgCl2, 0.5% Triton, 0.1% Tween-20, 1:5000 anti-foam). One-fifth of the beads were reserved for Western Blotting and remaining beads were resuspended in elution buffer (10mM EDTA, 50mM Tris-HCl, 1% SDS) and incubated at 65°C for 10min. Additional elution buffer containing TE+0.67% SDS was added to the beads and further incubated at 65°C for another 15min before addition of 100ug Proteinase K was added and the reactions were incubated at least 6 hours at 37°C. Eluted DNA from the IP and Input fractions were then collected by PCI and Ethanol precipitation for PCR.

Silver Stain

Proteins were resolved in SDS-PAGE and gels were subsequently fixed with 50% Ethanol 2X for 15min. Fixed gels were then treated with 5ug/ml DTT for 20min and 0.1% AgNO3 for 20min and finally developed using 3% Na2CO3(w/v) and 2% formaldehyde. The reaction was stopped by addition of 1% acetic acid and gels were imaged.

Spot growth assay

Yeast were pre-cultured to saturation overnight, diluted, and 1:10 serial dilutions were made with the most concentrated wells representing a concentration of 0.25 OD/ml. 3 mL of each dilution was spotted on YPD and grown at indicated temperatures for 2 days.

Live Cell Imaging

Imaging experiments were carried out using the confocal configuration of an Andor Dragonfly microscope equipped with an EMCCD camera driven by Fusion software (Andor) using a 60x oil immersion objective (Olympus, numerical aperture (N.A.) 1.4). Images were acquired from cells grown to mid log phase in SC media at 25°C and shifted to 37°C for indicated time or treated with DMSO or 500uM Auxin and 10 uM InsP6 for relevant experiments. Cells were immobilized in 384-well glass bottomed plates (VWR) that were pre-treated with Concanavalin A.

tRNA extraction and Northern Blotting

Isolation of small RNAs and Northern blot experiments were performed as previously described [176]. Briefly, small RNAs were isolated from yeast cultures grown to early log phase by addition of equal volumes cold TSE buffer (0.01M Tris pH7.5, 0.01M EDTA, 0.1M sodium chloride) and TSE saturated phenol. Samples were incubated at 55°C for 20min with vortexing every 3 min then placed on ice for 10 min. Aqueous phase was extracted after centrifugation, re-extraction with phenol was performed, and RNA was precipitated overnight in ethanol at -80°C. 2.5ug of RNA was then separated on 10% TBE-Urea gels, gels were stained with Apex safe DNA gel stain to visualize 25S and 18S rRNA and then transferred to Hybond N^+ membrane (Amersham). RNA was crosslinked to membranes at 2400J/m^2 using UV Crosslinker (VWR) and tRNA were detected using digoxigenin-labeled (DIG) probes. Mean integrated intensity values were measured for I and P bands using FIJI [177] and normalized to background in each lane.

Sequence of Northern blot probe targeting precursor and mature isoforms of $tRNA^{Ile}_{UAU}$ used in Figure 2 and 4 is as follows:

GGCACAGAAACTTCGGAAACCGAATGTTGCTATAAGCACGAAGCTCTAACCACTGAGCTACACGAGC.

Fluorescence In-Situ Hybridization (FISH)

FISH experiments were carried out as described in previous publications with minor modifications [178] using directly Cy3 labeled tRNA probes or fluorescein isothiocyanate (FITC) labeled dT probes. Sequence of Cy3 labeled $tRNA^{Ile}_{UAU}$ intron specific probe is same as previously published SRIM03 [179] with Cy3 moiety appended at the 5´ end (CGTTGCTTTTAAAGGCCTGTTTGAAAGGTCTTTGGCACAGAAACTTCGGAAACCGAATGTTGCTAT). Briefly, yeast cultures were grown to early log phase at 25°C and treated with drug/vehicle or shifted to 37°C for 4 hours when appropriate. Samples were fixed with 0.1 volume of 37% formaldehyde for 15min. These samples were then pelleted and resuspended in 4% paraformaldehyde (PFA) solution (4% PFA, 0.1M Potassium Phosphate (pH 6.5), 0.5mM $MgCl_2$) and incubated with rotation at 25°C for 3 hours. Cells were then washed twice with Buffer B (1.2M Sorbitol, 0.1M Potassium Phosphate (pH 6.5), 0.5mM $MgCl_2$). For spheroplasting cell pellets were resuspended in a 1ml Buffer B solution containing 0.05% beta-mercaptoethanol. 15ul of 20mg/mL zymolyase T20 was then added to each sample and were subsequently incubated at 37°C for 45min. After washing once with Buffer B, cells were adhered to eight well slides (Fisher Scientific) pre-coated with Poly-L-Lysine. Slides were placed sequentially in 70%, 90%, and 100% ethanol for 5min and then allowed to dry. Samples were next incubated at 37°C

for 2 hours in pre-hybridization buffer (4X SSC, 50% formamide, 10% dextran sulfate, 125 ug/mL *E. coli* tRNA, 500 ug/mL salmon sperm DNA, 1x Denhardt's solution, and 10mM vanadyl ribonucleoside complex (VRC)). Pre-hybridization solution was then aspirated and replaced with pre-warmed pre-hybridization solution that contained either 0.25uM Cy3 tRNA probe or 0.025uM FITC dT probe and further incubated at 37°C overnight. Wells were then sequentially washed for 5min once with 2x SSC, three times with 1xSSC, and once with 0.5x SSC. Slides were dipped in 100% ethanol, air-dried, and mounting media with 4', 6-diamidino-2-phenylidole (DAPI) was applied to each well prior to sealing of coverslip onto slide. Imaging was performed using Andor Dragonfly equipped with Andor iXon Ultra 888 EMCCD camera driven by Fusion software (Andor) using a 60x 1.4 N.A. oil objective. Images were processed in FIJI (e.g., cropping, brightness/contrast adjustments and maximum z-projections) [177]. Quantification of tRNA FISH was performed by acquiring average nuclear and cytoplasmic pixel intensities respectively. Maximum projection images were generated from z stack images for DAPI and tRNA FISH channels. Nuclear and whole cell masks were then respectively generated using Cellpose software [180, 181]. Nuclear mask regions were removed from whole cell masks to generate cytoplasmic mask and average pixel intensities were calculated. The ratio of average nuclear and cytoplasmic mask pixel intensity per cell was calculated.

RNA Immunoprecipitation (RIP)

RIPs were conducted as previously described with minor modifications [182]. Pull downs were performed targeting protein-A (prA) tagged Dbp5 in parallel with an untagged control to assess

background non-specific binding. Crosslinking was conducted on yeast cultures in mid-log growth by addition of formaldehyde to final concentration of 0.3% and incubation for 30 min. Crosslinking was quenched by addition of glycine to a final concentration of 60mM for 10 min. Cells were harvested and pellets frozen in liquid nitrogen. Lysis was performed using ice cold TN150 (50mM Tris-HCl pH7.8, 150mM NaCl, 0.1% IGEPAL, 5mM beta-mercaptoethanol) supplemented with 10mM EDTA, 1ng Luciferase Spike-In RNA (Promega), and 1X protease inhibitor cocktail. 1ml zirconia beads (0.5mm) were used for disrupting cells by vortexing 5 times for 30 seconds followed by 1min on ice between each pulse. Lysate was pre-cleared by centrifugation at 4000xg 5min followed by 20min at 20000xg at 4°C. 1% pre-IP lysate was preserved as input RNA sample and additional pre-IP sample was reserved for western blotting. Remaining lysates were then diluted to 10ml with TN150 and incubated with IgG-conjugated magnetic dynabeads at 4°C for 30min with constant rotation. Immunoprecipitate was washed once with 1ml TN150, once with 1ml TN1000 (50mM Tris-HCl pH7.8, 1M NaCl 0.1% IGEPAL, 5mM beta-mercaptoethanol), and again once with 1ml TN150 each for 5min at 4°C. Beads from RIPs were then resuspended in 500ul proteinase K elution mix (50mM Tris-HCl pH 7.8, 50mM NaCl, 1mM EDTA, 0.5% SDS, 100ug proteinase K) in parallel with input samples and incubated at 50°C for 2 hours followed by 65°C for 1 hour to allow crosslink reversal. RNA was isolated by extraction with acidic Phenol:Chloroform:Isoamylalcohol (pH 4.3-4.7) and ethanol precipitation. Half volume of RNA was reverse transcribed using Superscript III using random priming according to manufacturer instructions and other half of RNA was retained for minus RT control. RNA was analyzed by qPCR using Power SYBR (Applied Biosystems) on an Applied Biosystems instrument. Target RNA abundance in RIP was normalized to abundance of target in input sample and relative

fold enrichment was calculated by comparing signal in specific prA-Dbp5 RIPs to signal from untagged control. Standard curves were generated to test PCR efficiencies of each primer set used in this study. Primer sequences used are as follows:

$tRNA^{Ile}_{UAU}$ Unspliced Forward: GCTCGTGTAGCTCAGTGGTTAG

$tRNA^{Ile}_{UAU}$ Unspliced Reverse: CTTTTAAAGGCCTGTTTGAAAG

FBA1 Forward: CGAAAACGCTGACAAGGAAG

FBA1 Reverse: TCTCAAAGCGATGTCACCAG

Western Blotting

~5 OD pellets were lysed in laemmli buffer and loaded onto 10% SDS PAGE. Protein transfer was performed onto nitrocellulose membrane using cold wet tank transfer protocol. Western blotting was performed with 1:5000 dilution of mouse monoclonal anti-DBP5 and 1:10000 dilution of mouse monoclonal anti-GAPDH (Thermo Fisher) primary antibody overnight at 4°C and 1:10000 anti-mouse DyeLight 650 secondary to detect protein.

Protein Purification, ATPase assays and Fluorescence Polarization

Protein purification, *in vitro* ATPase assays and fluorescence polarization using full-length Dbp5 performed as described previously [183-185]. Commercially available Poly (I:C), mixed yeast tRNA and yeast Phenylalanine tRNA were obtained from Sigma for use in indicated assays. For RNase T1 ATPase assays, indicated RNA samples were treated with 33 units of RNase T1 for 2 hours at 37°C prior to experiment. Fluorescence polarization experiments were performed using a

fluorescein (fl) labelled 16nt ssRNA (5'-fl-GGGUAAAAAAAAAAAA-3') in the presence of ADP•BeF_3 with increasing concentrations of unlabeled polyadenylic acid or yeast mixed tRNA titrated into the reactions. Fluorescence polarization and ATPase assays were assembled in the same ATPase buffer (30mM HEPES [pH 7.5], 100mM NaCl, and 2mM $MgCl_2$). All ATPase assays with RNA titrations were fit to Michaelis Menten or allosteric sigmoidal models (when Gle1/$InsP_6$ was added) in GraphPad Prism.

RNA Electrophoretic Mobility Shift Assay (EMSA) and denaturing urea PAGE

Recombinant full length Dbp5 was purified as above and described previously[183, 185]. RNA binding and EMSA was performed according to previously published assay conditions [17]. RNA binding reactions were assembled using binding buffer (20mM HEPES [pH7.4], 100 mM KCl, 10 mM NaCl, 4 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5% NP-40) in the presence of indicated nucleotide and loaded on to 6% native polyacrylamide el (0.5 x TBE). Gels were stained with Apex gel stain to visualize RNA. Mean integrated intensity of bands were quantified in FIJI [177] and normalized to background signal for each respective well. Bound fraction was calculated and fit to a one-site binding equation in GraphPad Prism.

Chapter 3: Gle1 is required for tRNA to stimulate Dbp5 ATPase activity *in vitro* and to promote Dbp5 mediated tRNA export *in vivo*

3. 1. Introduction

Key to the production of proteins is the delivery of amino acids to ribosomes by transfer RNAs (tRNAs). While non-coding RNA (ncRNA) and mRNA export pathways have long been hypothesized to be regulated by independent processes, recent studies indicate the participation of numerous mRNA export factors in regulation of diverse ncRNAs [17, 19-21, 186-188]. In addition to tRNA and mRNA, both Mex67 and Crm1 have functions in pre-snRNA, pre-ribosomal subunit, and *TLC1* (telomerase) export [17, 19-21, 187, 188]. Similarly, in addition to Mex67 and Crm1, the DEAD-box Protein 5 (Dbp5) has also been implicated in each of these RNA export pathways, including tRNA export [21, 182, 187, 189, 190]. Dbp5 is most well known as an RNA-stimulated ATPase that is spatially regulated by the nucleoporins Nup159 and Gle1 with the small molecule inositol hexakisphosphate ($InsP_6$) at the cytoplasmic face of the nuclear pore complex (NPC) to promote the directional export of mRNA [108, 183, 184, 191-209]. In addition to these export roles, Dbp5 also shuttles between the nucleus and cytoplasm with reported roles in transcription, R-loop metabolism, and translation [182, 190, 193, 210-214]. Notably, Nup159 and Gle1 mutants also alter tRNA export [182], suggesting the mRNA export pathway as a whole (e.g., Mex67, Dbp5, Gle1/ $InsP_6$, Nup159) may support tRNA export. However, a mechanistic understanding of how Dbp5 supports these diverse functions and whether they are regulated by similar co-factors and enzymatic activity is largely not understood.

A recent mutagenesis screen within our research group identified mutants that alter the nucleocytoplasmic shuttling dynamics of Dbp5 [182]. Mutations were identified (e.g., *dbp5*^{L12A}) that disrupt a nuclear export sequence (NES) recognized by Crm1 to promote Dbp5 transport out of the nucleus. In contrast, another mutant, *dbp5*^{R423A}, was impaired in nuclear import from the cytoplasm. Importantly, neither of these mutations disrupt the essential mRNA export functions of Dbp5; however, limited nuclear access of *dbp5*^{R423A} induced tRNA export defects suggesting a potentially novel role for nuclear Dbp5 in tRNA export [182].

In this study, genetic and biochemical characterization of Dbp5 mediated tRNA export was performed. The data show that Dbp5 is recruited to pre-tRNA independent of Los1, Msn5, and Mex67, positioning the DEAD-box protein to function parallel to the primary Los1 mediated pre-tRNA export pathway. In contrast to single stranded RNA substrates (e.g., mRNA), this study further demonstrates an interaction of Dbp5 with tRNA and double stranded RNA (dsRNA) that leads to robust ATPase activation only in the presence of Gle1/ $InsP_6$. These findings, together with previous research [182], suggest that Dbp5 engages tRNA within the nucleus in a manner distinct from mRNA, and requires Gle1/ $InsP_6$ to mediate RNA stimulated ATPase activation of Dbp5 to promote export.

3. 2. Results

3. 2. 1. Dbp5 directly binds pre-tRNA in-vivo

Many DEAD-box proteins function both directly and indirectly in various steps of gene regulation. Given the tremendous regulatory feedback between different stages of gene expression, and the many proposed roles of Dbp5, it is challenging to identify direct functions of the protein without knowing its RNA targets *in-vivo*. For this reason, we generated an atlas of

RNA substrates of Dbp5 using Crosslinking and Analysis of cDNAs (CRAC). Autoradiographs confirm that following a two-step immunoprecipitation (prA then His6) and separation on SDS PAGE, Dbp5 co-immunoprecipitates crosslinked RNA that migrates at the molecular weight of the protein (Figure 3-1A). This indicates RNA isolated from CRAC is likely specific to Dbp5 and not resulting from contaminating proteins in Dbp5 IPs. Sequencing from a single replicate of CRAC for Dbp5, *dbp5*^{L12A}, *dbp5*^{R423A} and an untagged background control consisted of mRNAs and an abundance of rRNA (a common contaminant in CRAC experiments, but also a published target for Dbp5 regulation) (Figure 3-1B) [122, 143, 165, 214]. However, one class of RNA that made up a small fraction of reads in the background control but sizable portion of hits in Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} IPs was tRNA (Figure 3-1B). Despite the cellular abundance of tRNAs, these transcripts are not frequently observed to physically interact with RBPs unless the protein participates in tRNA biology [161, 215, 216].

We confirmed a physical interaction with tRNA was specific and not an artifact of UV crosslinking or UV induced DNA damage response by using formaldehyde crosslinking and RNA IP (Figure 3-1C-E). Formaldehyde crosslinked Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} purified using same stringent wash conditions as CRAC yielded IPs free of contaminating proteins, as measured by silver staining and Western blotting (Figure 3-1C). By RNA IP, Dbp5 shows a ~10-fold enrichment for pre-tRNA$^{Ile}_{UAU}$ above background non-specific IP (untagged Dbp5 strain). While *dbp5*^{R423A} has a slight reduction in average fold enrichment of pre-tRNA, *dbp5*^{L12A} causes a significant increase in Dbp5-tRNA interactions (~10-fold to ~100-fold enrichment) (Figure 3-1E). *Cox1,* one of the most abundant mitochondrial mRNA transcripts in yeast, is not enriched in Dbp5, *dbp5*^{L12A} or *dbp5*^{R423A} IPs consistent with the fact that Dbp5 is not predicted to function in mitochondrial

biology (Figure 3-1D) [217]. This importantly confirms RNA targets not predicted to be regulated by Dbp5 do not co-immunoprecipitate by this assay. Together these results suggest that Dbp5 physically interacts with pre-tRNAs and that elevating nuclear pools of the protein further increases this interaction, suggesting a nuclear role for Dbp5 in pre-tRNA processing and/or export.

3. 2. 2. Dbp5 functions parallel to Los1 in pre-tRNA export

The β-importins Los1 and Msn5 function in transport of both precursor and spliced tRNA (Los1) or specifically mature amino-acylated tRNA export (Msn5) pathways respectively [80-82, 218]. The viability of the *los1Δ*/*msn5Δ* double mutant has prompted investigations of additional proteins that promote tRNA transport which have resulted in multiple mRNA export factors being identified to co-function in tRNA export [63, 85, 86]. Previous studies have placed the mRNA export factor Mex67, a proposed target of Dbp5 regulation in mRNA export [198, 209], in a pathway parallel to Los1 regulated pre-tRNA nucleocytoplasmic shuttling [179]. To investigate the relationship between Dbp5, Los1, and Msn5 respectively it was first determined if the subcellular distribution of either protein was dependent on the activity of the other. In *los1Δ* and *msn5Δ* respectively, the NPC localization of GFP-Dbp5 was not altered (Figure 3-2A). Similarly, when a Dbp5 loss of function was induced using Auxin Induced Degradation (AID) [219], Los1-GFP and Msn5-GFP localization remained unchanged (Figure 3-2B). Under these same conditions mRNA export defects were detected (Figure 3-2C), confirming efficient depletion of Dbp5 to a level sufficient to disrupt mRNA export and induce loss-of-function. To test the genetic and phenotypic relationship of Los1, Msn5, and Dbp5, either *los1Δ* or *msn5Δ* was combined with the previously identified Dbp5 shuttling mutants (i.e., $dbp5^{L12A}$ and $dbp5^{R423A}$) in a yeast shuffle strain

where the sole copy of Dbp5 is provided by a CEN plasmid. Like a *mex67-5/los1Δ* strain [179], double mutants of *los1Δ* with *dbp5*^{L12A} (increased nuclear Dbp5) or *dbp5*^{R423A} (depleted nuclear Dbp5) exhibited no growth defects (Figure 3-2D) while *msn5Δ/dbp5*^{R423A} show a slight synthetic growth defect at 37°C. In contrast, by tRNA FISH using probes that detect both mature and un-spliced isoforms of tRNA$^{Ile}_{UAU}$ (SRIM04), *msn5Δ/dbp5*^{R423A} exhibit no synthetic phenotype on export status, while *los1Δ/dbp5*^{R423A} show an additive nuclear accumulation (Figure 3-2E/F).

To complement the FISH data, Northern blotting analyses were conducted to assess pre-tRNA processing in the double mutants. Intron-containing pre-tRNAs are transcribed with 5′ leader and 3′ trailer sequences to generate precursor molecules (P) that are end-processed in the nucleus to generate intron-containing intermediates (I) that can be detected by Northern blotting [85]. When nuclear export of the end-processed pre-tRNA is disrupted a change in the precursor (P) to intron-containing intermediate (I) ratio is observed [85, 176, 179]. Consistent with tRNA FISH, Northern blots revealed an additive defect in precursor tRNA export in *los1Δ/dbp5*^{R423A} shuffle strains (Figure 3-2G).

To further validate the growth and phenotypic characterization of *los1Δ/dbp5*^{R423A} double mutants, the mutations were integrated into yeast at their endogenous gene loci. Consistent with the shuffle strains, no synthetic genetic interaction was detected between Dbp5 and Los1 when double mutants were endogenously expressed, but significant additive defects in precursor tRNA export were observed by FISH and Northern blotting (Figure 3-3A-F). Given the observed precursor tRNA export defect detected by Northern blotting in *los1Δ dbp5*^{R423A} double mutants (~2-fold increase in the I/P ratio relative to *los1Δ* alone, Figure 3-3B/C), tRNA FISH was repeated using a probe specific to intron containing pre-tRNA$^{Ile}_{UAU}$ (SRIM03). After 4-hour incubation at

37°C the *los1Δ dbp5*^{R423A} double mutant showed significantly stronger nuclear accumulation of pre-tRNA as compared to single mutants or control (Figure 3-3E/F). Additionally, in *los1Δ dbp5*^{R423A} pre-tRNAs accumulated in a structure adjacent, but not overlapping the DAPI staining, likely indicating a nucleolar accumulation of pre-tRNAs in the mutant. This may be reflective of an early nuclear role for Dbp5 in pre-tRNA export that contrasts its terminal functions in mRNA transport. Notably, elevating nuclear pools of Dbp5 by combining *dbp5*^{L12A} with *los1Δ* in shuffle and integrated mutant strains did not alter the pre-tRNA export defects observed in *los1Δ* strains, indicating that excess nuclear Dbp5 is not sufficient to suppress the *los1Δ* phenotype.

One possible explanation for an additive pre-tRNA nuclear accumulation in a *los1Δ dbp5*^{R423A} double mutant could be a disruption of Dbp5 mediated transcriptional repression of tRNA genes. If nuclear Dbp5 has a transcriptional repression role similar to the RNAPIII regulator Maf1, limiting nuclear pools with *dbp5*^{R423A} could hypothetically result in saturation of existing export machinery and accumulation of nuclear pre-tRNAs. Genetic interactions between Dbp5 and transcriptional machinery (RNAPII) have been previously identified by large scale screens [151, 196]. Importantly Maf1 nucleocytoplasmic shuttling and function is also regulated by Msn5 which shows a weak genetic interaction with *dbp5*^{R423A} (Figure 3-2D). To test for a genetic interaction between Dbp5 and RNAPIII transcription, double mutants of Maf1 and Dbp5 were generated in the previously described Dbp5 shuffle strains. The *maf1Δ dbp5*^{R423A} double mutant, like *msn5 dbp5*^{R423A}, showed a weak negative genetic interaction at 37°C, but no strong synthetic growth phenotype in *maf1Δ dbp5*^{L12A} was observed (Figure 3-4A). Given the weak genetic interaction, we tested a direct role in transcription by performing Dbp5 ChIP using a previously published multiplex PCR strategy that has successfully detected RNAPIII and Maf1 at tRNA gene

loci [174, 175]. As a positive control Rpc82 (subunit unique to RNAPIII) was used to confirm chromatin enrichment at tRNA genes. PCRs were multiplexed with primers targeting a control region, TRA1, an mRNA gene locus that is regulated by RNAPII and not a predicted target of Rpc82. Additionally non-specific IPs were also conducted in strains not expressing the epitope tag (protein A) used for specific pulldown of Rpc82 and Dbp5. Enrichment for Rpc82 and Dbp5 could be detected by western blotting and chromatin enrichment could also be detected in Rpc82 pulldowns, however no such enrichment at tRNA gene loci could be confirmed for Dbp5 (Figure 3-4B/C). A lack of physical interaction does not preclude a possible direct interaction with transcriptional regulation. However, these results are consistent with multiple failed efforts to detect Dbp5 at RNAPII gene loci despite genetic and physical interactions with RNAPII regulatory machinery [151, 153, 196]. Future studies will likely elucidate the nature of direct and/or indirect Dbp5 function in transcription.

To test if the additive accumulation of end processed pre-tRNAs in *los1Δ dbp5*^{R423A} mutants is indeed caused by a transcription independent export role, we monitored pre-tRNA export kinetics in *los1Δ* and *los1Δ dbp5*^{R423A} following thiolutin treatment using Northern blotting. Upon thiolutin treatment, pre-tRNA with 5' leaders and 3' trailer sequences ("P" bands) can no longer be detected due to ongoing processing, and over time end processed intermediates ("I" bands) will also disappear due to ongoing export in the absence of new transcript production. In a *los1Δ dbp5*^{R423A} mutant end processed pre-tRNAs persist at 30 and even 60min after thiolutin treatment in contrast to *los1Δ* alone which shows no pre-tRNAs even after 30min thiolutin treatment. This observation is supportive of delayed kinetics of pre-tRNA export in the absence of ongoing transcription in a *los1Δ dbp5*^{R423A}.

Given the well characterized role of Dbp5 as an mRNA export factor and analysis presented here, we propose that Dbp5 functions in pre-tRNA export in a molecular pathway that functions parallel to Los1. Strikingly the phenotypic characterization of Dbp5 strongly mirrors the published role for Mex67 mediated regulation of pre-tRNA export, which also functions parallel to Los1 [63].

3. 2. 3. Known tRNA export factors do not recruit Dbp5 to pre-tRNAs in vivo

To better characterize the composition of Dbp5 containing tRNPs, co-immunoprecipitation experiments were employed to determine if known tRNA export factors form larger macromolecular complexes with Dbp5. Previous studies have suggested Dbp5 forms physical interactions with Mex67 and Crm1, which both function in mRNA and tRNA export [88, 126, 209]. We extended this analysis to nuclear tRNA export factors. In addition to Los1 and Msn5, we tested for physical interactions with the nucleolar factors Utp8 and Utp9 as well. Nucleolar pools of Dbp5 have been reported and Utp8 and Utp9 have been implicated in tRNA export functions upstream of Los1 and Msn5 through an unknown mechanism. Additionally nucleolar accumulation of pre-tRNAs in a *los1Δ dbp5*^{R423A} mutant may suggest an early role for Dbp5 in tRNA biogenesis. Additionally, Utp17, a nucleolar protein that is not known to be involved in tRNA processing, was also tested as a negative control. All IPs were conducted in parallel with a non-specific IP from a lysate not expressing the epitope tag used for Dbp5 pulldowns. Furthermore, IPs were conducted after growth in both rich media and nutrient starvation conditions where tRNA nucleocytoplasmic shuttling is highly regulated. Utp8 and 9 interactions could be readily detected under conditions of rich growth (Figure 3-5C/D). However, all tested factors were enriched and/or had elevated interaction with Dbp5 under conditions of amino acid

starvation, and to a lesser extent, glucose starvation (Figure 3-5A-E). Elevated physical interactions between tRNA export factors and Dbp5 under these conditions may be reflective of tRNA export factors being sequestered under export repressive conditions or indicative that multiple export factors from independent pathways co-occupying nuclear tRNPs together. Importantly, Utp17 enrichment may indicate that Dbp5 is broadly interacting non-specifically with nucleolar/nuclear proteins given its localization. Alternatively, these results could be reflective of an uncharacterized role for Utp17 in tRNA export.

Dbp5, like other DEAD-box proteins, has been reported to bind nucleic acids through sequence independent interactions with the phosphate backbone [139, 183, 220, 221]. For other helicases of the SF2 family, specificity in RNA substrates is conferred by adaptor proteins [198, 222, 223]. For this reason, it was investigated whether known tRNA export factors mediate recruitment of Dbp5 to pre-tRNA substrates. To do so, RNA immunoprecipitation (RIP) experiments were performed with protein-A (prA) tagged Dbp5, integrated at its endogenous locus and present as the sole copy of the gene, in strains where tRNA export factors were deleted or depleted. Co-immunoprecipitated RNAs were analyzed by RT-qPCR with primers specific to un-spliced intron-containing pre-tRNA$^{Ile}_{UAU.}$ The abundance of the pre-tRNA$^{Ile}_{UAU}$ target in each IP was normalized to the abundance of the target in the corresponding input sample to control for changes in gene expression. Relative enrichment of the target RNA was then compared to the background signal obtained from RNA IPs in a common untagged control. Given the observed physical interactions under rich growth, we first tested whether essential nucleolar factors Utp8 and 9 facilitate Dbp5 recruitment to tRNA. To do so, Auxin Induced Degrons were used to deplete the factors prior to IP (Figure 3-6A/B). At both 30 and 60 minutes neither factor was able to

abolish Dbp5-tRNA interaction upon loss-of-function despite efficient degradation of Utp8 and Utp9 (Figure 3-6A/B).

We next tested non-essential tRNA export factors Los1 and Msn5 as adapters by deleting genomic copies of the genes. In a *los1Δ* strain, a ~2-fold reduction in the relative amount of pre-tRNA co-immunoprecipitated with Dbp5 (~9.5-fold enrichment in WT to ~4.5-fold in *los1Δ*) was observed, but the deletion also failed to abolish the Dbp5 pre-tRNA *in vivo* interaction (Figure 3-6C). Loss of Msn5, which does not have a published role in the export of intron-containing pre-tRNAs [82], did not cause a significant change in Dbp5 pre-tRNA interactions by RNA IP either (Figure 3-6C). These results are consistent with the additive tRNA export defects observed in *los1Δ dbp5*^{R423A} double mutants reported in Figure 3-2/3-3 and support the hypothesis that Dbp5 functions in tRNA export parallel to Los1.

In mRNA export, Mex67 is a proposed target of Dbp5 activity to promote directional nuclear export [209]. Since neither of the non-essential tRNA export factors or Utp8/9 abolished Dbp5-tRNA interaction when depleted, it was tested if Mex67 has a role in recruiting Dbp5 to tRNA. An Anchors Away approach was used to rapidly re-localize Mex67 to the cytoplasmic peroxisomal anchor (Pex25-FKBP12) in an inducible manner dependent on the addition of rapamycin [224]. The resulting strains were sensitive to rapamycin, causing nuclear mRNA accumulation by dT FISH within 15 minutes of addition (Figure 3-6F). This timing is consistent with a previous report employing Mex67 Anchors Away (Mex67-AA) [225]. PrA-Dbp5 was integrated at its endogenous locus in these strains and RNA-IP experiments were performed before and after rapamycin addition as described above. Under these conditions of Mex67-AA re-localization, no significant change in the Dbp5-tRNA$^{Ile}_{UAU}$ interaction was observed (median of

~8.8-fold enrichment before addition of rapamycin and ~8.2-fold after, Figure 3-6D). Importantly, consistent with the function of Mex67 in mRNA export [191, 192, 194, 195, 197, 198, 200, 202, 203, 209], the nuclear retention of mRNAs via Mex67-AA resulted in strongly reduced binding of Dbp5 to the *FBA1* mRNA (from a median of ~8 fold enrichment above background before addition of rapamycin to no enrichment after, Figure 3-6E).

In rRNA export, loops in Mex67 NTF2-like domains facilitate a direct interaction between Mex67 and pre40S ribosomal subunits to promote export [17-19]. Mutations in these residues were tested for tRNA processing as a means of assessing whether Mex67 can bind and regulate tRNA in the absence of an adapter protein. Indeed, none of these perturbations (*mex67Δloop, mex67K343E, mex67Δloop K343E, or mex67 KR→AA*) induced observable tRNA export defects by Northern blotting at 25°C and 37°C (Figure 3-6G). In addition, Yra1 AID was tested for defects in tRNA processing. Yra1 serves as an adapter to facilitate Mex67 recruitment to mRNA and has never been tested for a role in tRNA processing despite large scale screens of mutants in nearly all known yeast genes [85]. Despite inducing strong mRNA export defects, tRNA export was largely unaffected in these mutants as well (Figure 3-6H). Together, these co-IP, Northern blotting and FISH experiments suggest that Dbp5 binds to tRNAs independent of known tRNA export proteins, Mex67 seemingly requires an adapter for recruitment to tRNA, and the recruitment of these factors to tRNA is distinct from mechanisms that regulate mRNP and pre-ribosomal subunit formation and export.

3. 2. 4. The Dbp5 ATPase cycle supports tRNA export in vivo

In mRNA export, RNA binding, Gle1 activation, and ATP hydrolysis by Dbp5 are all critical for directional transport [204, 205]. The *dbp5*^{R426Q} and *dbp5*^{R369G} mutants exhibit deficient RNA

binding (<5% of WT), ATPase activity (~10% and 60% of WT, respectively) and have a dominant-negative effects on mRNA export status when overexpressed [204, 205]. Additionally, *dbp5*^{R369G} has elevated Gle1 affinity and effectively competes with WT Dbp5 for Gle1 binding [205]. In contrast, *dbp5*^{E240Q} is an ATP hydrolysis mutant that is competent to bind RNA, as it is biased to the ATP bound state, but is recessive lethal showing no mRNA export defect when overexpressed in the context of endogenously expressed WT Dbp5 [205]. Unlike the impact of these mutants on mRNA export, the ability of Dbp5 to complete ATP hydrolysis (but not RNA binding) is dispensable for pre-ribosomal subunit export, as is Gle1 [189].

Based on the observation that Gle1 functions to support tRNA export [182], these Dbp5 mutants deficient in ATPase activity or altered Gle1 binding were tested for their influence on tRNA export to infer if tRNA export resembles mRNA or pre-ribosomal subunit export. To individually express these lethal mutants and the wildtype control, *DBP5*, *dbp5*^{R426Q}, *dbp5*^{R369G}, or *dbp5*^{E240Q} were integrated at the *URA3* locus under regulation of the inducible pGAL promoter. Protein expression was induced for 6 hours by shifting cells from raffinose to galactose containing media. Importantly, induction was followed by 1 hour of growth in glucose to halt expression and relieve potential changes in tRNA export induced by a change in the primary carbon source. Using this approach, all proteins were expressed as indicated by western blotting (Figure 3-7A) and showed the previously reported impact on mRNA export (Figure 3-7B). Northern blotting targeting $tRNA^{Ile}_{UAU}$ revealed both *dbp5*^{R426Q} and *dbp5*^{R369G}, but not *dbp5*^{E240Q}, showed an accumulation of intron-containing precursor $tRNA^{Ile}_{UAU}$ as compared to overexpression of wildtype Dbp5 (I/P ratio of 1.97 +/- 0.06 vs 1.93 +/- 0.31 vs 1.32 +/- 0.41, respectively) (Figure 3-7C/D). These phenotypes differ from the reported impacts on pre-ribosomal subunit transport,

indicating Dbp5 functions differently in these non-coding RNA export pathways. Furthermore, these data indicate that both the ATPase cycle and regulation of Dbp5 by Gle1 are central to the function of Dbp5 in tRNA export, as each activity is in mRNA export.

3. 2. 5. Dbp5 ATPase activity in the presence of tRNA is Gle1-dependent

Given the impact of *dbp5*^{R426Q} and *dbp5*^{R369G} overexpression on tRNA export *in vivo*, the Dbp5 ATPase cycle and role of Gle1 in the context of tRNA was further investigated *in vitro*. Previous studies have characterized Dbp5 binding to single stranded RNA (ssRNA) substrates and demonstrated Dbp5 binding to ssRNA is linked to the nucleotide state of the enzyme, with the highest affinity for ssRNA occurring when Dbp5 is ATP bound [183, 184, 226]. Following ssRNA binding, hydrolysis of ATP to ADP results in a reduction in the affinity of Dbp5 for the ssRNA substrate [184]. While there has been extensive characterization of the structural and biochemical details of Dbp5, ssRNA, and co-regulators [208, 227, 228], no such understanding exists for Dbp5 engaging highly structured RNA substrates like tRNA. As such, it was first tested if recombinant full length Dbp5 could bind commercially available yeast mixed tRNAs or the yeast Phenylalanine tRNA (both substrates that have been used in previous biochemical and structural studies [17, 57]). To test whether Dbp5 can form complexes with tRNA *in vitro,* electrophoretic mobility shift assays (EMSA) were performed in the presence of different nucleotide states and tRNA substrates. Consistent with published observations of complex formation between Dbp5 and ssRNA [183, 184], Dbp5 generated band shifts for both mixed tRNA substrates and Phe tRNA in the presence of the ATP state analog ADP•BeF_3 (Figure 3-8A). In the presence of ATP or ADP, or with no nucleotide, Dbp5 failed to form a similar band shift, which exactly parallels reported interactions between Dbp5 and ssRNA in these nucleotide states [183, 184].

To further estimate an affinity for tRNA, both the Phenylalanine tRNA and yeast mixed tRNAs were used in EMSA experiments with varying concentrations of Dbp5 (and fixed concentrations of ADP•BeF_3 and tRNA). From quantification of the EMSAs, an estimated K_d of ~150 nM (Phe tRNA) and ~130 nM (mixed tRNA) was obtained (Figure 3-8B/C). In addition to EMSAs, Dbp5 binding to tRNA was observed and tested in competition assays using fluorescence polarization measurements. In these assays, increasing concentrations of unlabeled tRNA or a ssRNA homopolymeric polyadenylic acid (pA, routinely used for RNA activation in steady-state ATPase assays [184]), were found to compete with a fluorescently labeled ssRNA for Dbp5 in the presence of ADP•BeF_3 (Figure 3-8D). These data, from orthogonal approaches, indicate that Dbp5 interacts directly with tRNA *in vitro,* and does so in a manner governed by principles that resemble Dbp5-ssRNA binding.

Though Dbp5 is generally viewed as performing RNPase activity on mRNAs, rather than using traditional helicase activity to unwind duplexes, the ability of Dbp5 to unwind duplexed RNA was revisited due to the highly structured nature of tRNA. Dbp5 has unwinds short duplexed RNAs with 5' overhangs, however the influence of Gle1/$InsP_6$ on this activity has not been reported [104]. Fluorescence unwinding assays were employed to test if Gle1 can accelerate Dbp5 mediated duplex unwinding. Double stranded RNA (dsRNA) with 5' overhangs were generated by annealing unlabeled oligos with a complementary FAM labelled fluorescent RNA. Fluorescent dsRNA was combined with Dbp5 and RNase T1 and incubated at 37°C for indicated times. Because RNase T1 can only digest single stranded RNA (not dsRNA), duplex unwinding kinetics can be monitored by observing disappearance of fluorescent dsRNA. As previously reported, Dbp5 is only able to unwind duplexed dsRNA in the context of ATP in the absence of

co-regulators, with most dsRNA being unwound within 30min (Figure 3-9A) [104]. However, performing these experiments in the context of Gle1/$InsP_6$ did not appear to have an obvious effect on these kinetics (Figure 3-9B).

Interaction with ssRNA substrates has been well documented to stimulate the Dbp5 ATPase cycle [183, 184, 197-200, 202-205]. As such, it was next determined if binding of tRNA to Dbp5 stimulates ATPase activity using a spectrophotometric ATPase assay [183, 184]. Unlike a ssRNA substrate (i.e., pA) that can maximally stimulate ATP turnover to ~0.5 ATP/sec, neither tRNA nor poly(I:C) (used as an orthogonal dsRNA substrate) stimulated Dbp5 ATPase activity over a range of RNA concentrations (Figure 3-9C). These results with the above binding data suggest Dbp5 can engage structured and dsRNA substrates, but this does not lead to productive ATP hydrolysis.

The nucleoporin Gle1 with the small molecule inositol hexakisphosphate ($InsP_6$) synergistically activates Dbp5 ATPase activity at low RNA concentrations [183, 184]. These findings have led to a model of spatially regulated Dbp5 activity to promote mRNA-protein complex remodeling at the cytoplasmic face of an NPC where Gle1 is localized, resulting in directional mRNA export [183, 184, 193, 194, 199, 202, 204, 205, 207, 208, 226]. In the context of tRNA, addition of Gle1/$InsP_6$ maximally stimulated Dbp5 (1.03 +/- 0.04 ATP/s) to a level like that of ssRNA (1.11 +/- 0.07) (Figure 3-9D). The synergistic activation of Dbp5 ATPase activity by Gle1/$InsP_6$ and tRNA is mirrored by poly(I:C) and was observed with both mixed yeast tRNAs as well as yeast Phenylalanine tRNA (Figure 3-9D). To confirm that this enhanced RNA activation by Gle1/$InsP_6$ was not the result of contaminating ssRNA, ATPase assays were performed after treatment of tRNA or poly (I:C) with RNase T1 for 2 hours at 37°C. RNase T1 degrades ssRNA, not

dsRNA, allowing determination of whether RNA activation observed in the presence of Gle1/$InsP_6$ is specific to properly folded tRNA and poly(I:C). As expected, treatment of the ssRNA (pA) with RNase T1 resulted in the loss of RNA-stimulated Dbp5 ATPase activity (0.68 +/- 0.06 before RNase T1 treatment to 0.17 +/- 0.01 after). Furthermore, RNase T1 treatment reduced pA/Gle1/$InsP_6$ activation (0.98 +/- 0.17 before treatment to 0.69 +/-0.07 after) to that of Dbp5 and Gle1/$InsP_6$ alone (0.62 +/- 0.03) (Figure 3-9E). However, RNase T1 treated tRNA remained unchanged in the ability to activate Dbp5 in the presence of Gle1/$InsP_6$ (1.19+0.11 without vs 1.18+/-0.19 ATP/s with RNase T1 treatment). This was recapitulated with poly(I:C) (1.26 +/- 0.11 ATP/s without and 1.46 +/- 0.135 ATP/s with RNase T1), confirming that the synergistic activation of Dbp5 observed in the presence of Gle1/$InsP_6$ is dependent on the structured tRNA or dsRNA. These findings suggest the possibility that Dbp5 engages tRNA in the cell without ATPase activation and could remain bound until the Dbp5-tRNA complex reaches Gle1 at the cytoplasmic side of a nuclear pore complex.

3. 2. 5. Discussion

The best characterized tRNA export factors in *S. cerevisiae*, Los1 and Msn5, are non-essential and the *los1D/msn5D* double mutant is viable despite the essential role of tRNA export [83, 90]. These data suggest additional mechanisms for regulated tRNA export, which is supported by recent publications that have implicated mRNA and rRNA export factors such as Mex67, Nup159, Gle1, Dbp5, and Crm1 in tRNA export [63, 85, 95, 179]. However, a mechanistic understanding of how these factors function in tRNA export and how such roles differ or relate to previously characterized roles in RNA export is still unresolved. Here, a combination of *in vivo* and *in vitro* data provide evidence that (1) Dbp5 functions parallel to Los1 in tRNA export, similar

to the mRNA export factor Mex67 and Crm1; (2) Dbp5 can bind tRNA directly *in vitro* and does so independently of Los1, Msn5, and Mex67 *in vivo*; and (3) the Dbp5 ATPase cycle is uniquely modulated by Gle1 in the presence of structured RNA (e.g. tRNA or dsRNA) *in vitro* and the ATPase cycle supports tRNA export *in vivo*.

Collectively, the *in vitro* characterization of Dbp5 and tRNA described here suggest two non-mutually exclusive possibilities for why Dbp5 is only activated by tRNA/dsRNA in the presence of Gle1/$InsP_6$. First, it may be that Dbp5 binds tRNA to form an inhibited intermediate that is relieved by Gle1/$InsP_6$. This is an attractive hypothesis that is unique from how Dbp5 engages ssRNA (e.g., mRNA) and potentially supports previously proposed export models that suggest Dbp5 may bind and travel with a tRNA from nucleus to cytoplasm [182]. A second possibility is that Gle1 enhances the affinity of Dbp5 for tRNA and "non-mRNA" substrates, which is supported by biochemical characterizations that show Gle1 promotes a shift in the steady-state distribution of populated Dbp5 intermediates from weak to strong RNA binding states [183, 204-206, 208, 227]. Future structural and biochemical studies are expected to distinguish among these possibilities. Moreover, Dbp5 was previously reported to not bind dsRNA substrates [184], as such the findings that Dbp5 can bind, and in the presence of Gle1, be activated by tRNA should motivate reevaluation of possible functions of Dbp5-Gle1/$InsP_6$ in regulating other highly structured RNA or "non-canonical" substrates *in vivo*.

While it has been reported previously that tRNA fails to stimulate Dbp5 ATPase cycle [191], here it is shown that despite a lack of stimulation when Gle1/$InsP_6$ is absent, Dbp5 is able to bind tRNA *in vitro* and *in vivo*. The ability of a tRNA to bind but not stimulate the ATPase cycle of Dbp5 has important implications for mechanisms by which Dbp5 may function in tRNA export.

For example, in mRNA export it is thought that Dbp5 functions are localized at NPCs [182, 209, 229], where the Dbp5 ATPase cycle is tightly regulated by co-factors Nup159 and Gle1/$InsP_6$ [183, 193, 204, 205, 208, 230]. In fact, recent studies have shown the essential function of both Dbp5 and Mex67 in mRNA export can be accomplished when these proteins are fused to nuclear pore components, in addition Dbp5 does not form a complex with mRNAs in the nucleus [209, 229]. These reports suggest that Dbp5 does not travel with an mRNA from the nucleoplasm to the cytoplasmic face of an NPC as part of an mRNA-protein complex, rather Dbp5 likely engages mRNAs after they exit the NPC transport channel to direct the final step(s) of mRNA export (Figure 6). However, in contrast to this mRNA export model, the unique ability of Dbp5 to bind tRNA without ATP stimulation, and then be fully activated by Gle1/$InsP_6$, presents the potential for a novel mechanism of function in regulating tRNA export. Namely, that Dbp5 may enter the nucleus to stably engage tRNA and direct events leading to export, following which Dbp5 is removed from the tRNA by Gle1/$InsP_6$ upon nuclear exit (Figure 3-10). Importantly, in both mRNA and tRNA export, Gle1/$InsP_6$ activation is required to stimulate ATPase activity and recycle the enzyme. In contrast, for pre-ribosomal subunit export Dbp5 ATPase activity and Gle1 have been shown to be dispensable for transport [189]. This raises interesting questions of whether RNA binding and nucleocytoplasmic shuttling of Dbp5 alone can achieve this function, where in the cell these interactions occur, and how rRNA substrates modulate Dbp5 activity.

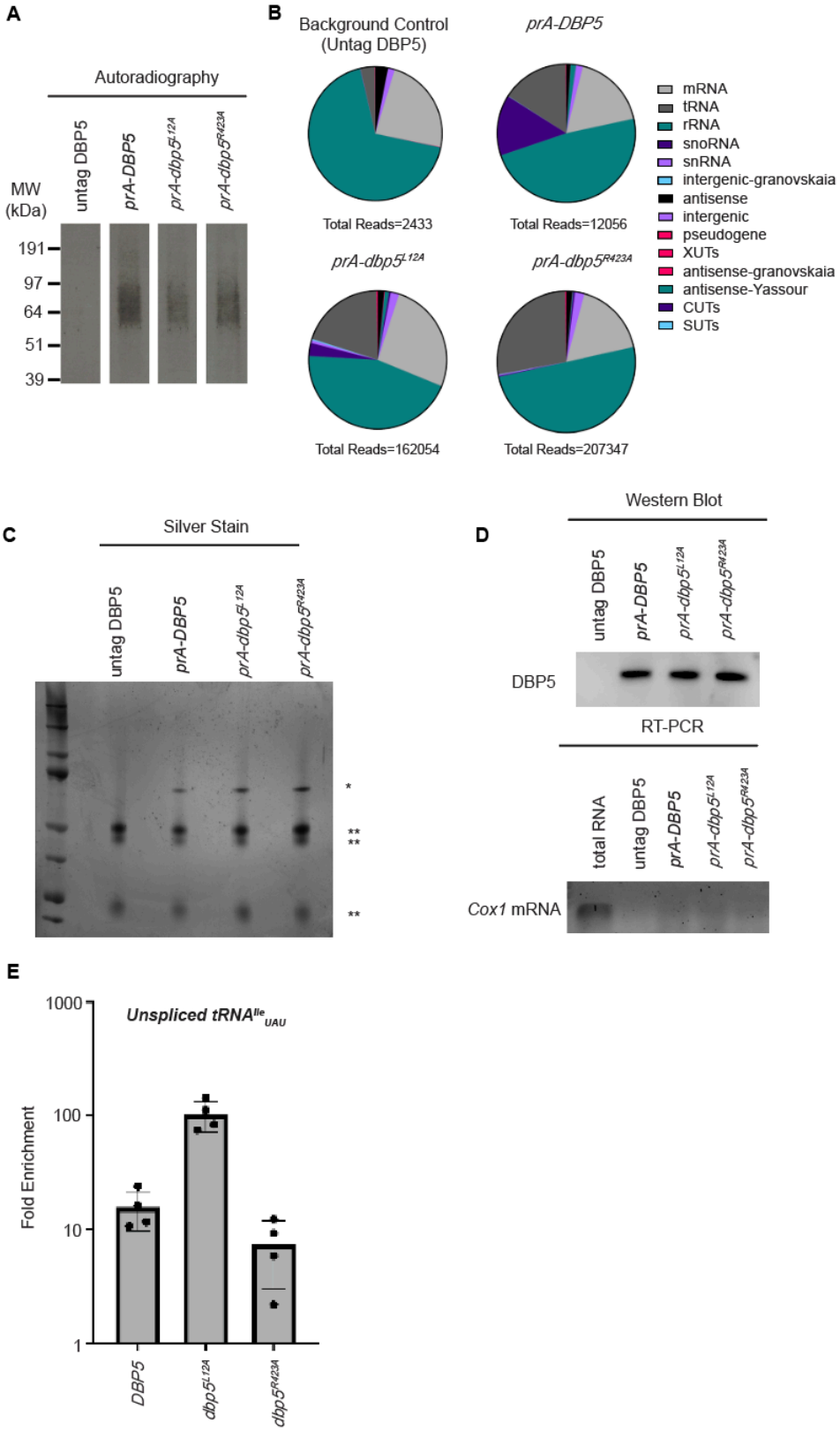
In mRNA export, it has been proposed that Dbp5 displaces mRNA export factors such as Mex67 and Nab2 to promote directionality of mRNA transit [198, 200]. Observations that Mex67 and Dbp5 appear to both function parallel to Los1 to promote tRNA export may point to an overlapping pathway that shares both mRNA export factors. Dbp5 and Mex67 have been

proposed to form physical interactions, with Mex67 serving as an adaptor to direct Dbp5 to appropriate mRNA sites for remodeling [198]. However, here it is demonstrated that Mex67 is not required for Dbp5-tRNA interaction based on RNA IP assays. Additionally, previously published data suggests Mex67 likely requires an unknown adaptor to form its reported interactions with its tRNA substrates [17]. Given the *in vitro* properties of Dbp5 binding to tRNA, it is tempting to speculate that nuclear Dbp5 promotes recruitment of Mex67 or Crm1 to tRNA-protein complexes. Indeed, a similar phenomenon has been described for the exon junction complex (EJC) and the DEAD-box protein at its core, eIF4AIII, that acts as a platform for the binding of other proteins to an mRNA [220, 221, 231, 232]. Stable binding of the EJC is governed by trans-acting proteins that lock the complex on the mRNA by stabilizing an ADP-P_i hydrolysis immediate [113, 221, 231, 233]. In the case of Dbp5, the *in vitro* data suggests an inhibited state may be achieved in the context of tRNA alone, which could be further stabilized *in vivo* by yet to be discovered protein factors. The ability of Gle1/$InsP_6$ to induce maximal activity of Dbp5 in the presence of tRNA would then allow for resolution of stable Dbp5-tRNA complexes in a spatially regulated manner to terminate export and recycle bound export factors (Figure 3-10). Alternatively, given that it has been reported that Mex67 can bind 5´ extended tRNAs and that these transcripts have been previously reported to contain 5´ methyl-guanosine cap structures [63, 66], perhaps an “mRNA export-like” pathway that involves Cap Binding Complex (CBC) can act to recruit Mex67 to a subset of tRNAs. It remains unclear if CBC interacts with such transcripts and whether other export factors like Dbp5 also support “premature” export of unprocessed tRNAs.

More broadly, like many DEAD-box proteins, Dbp5 has many reported roles in controlling gene expression [182, 187, 189, 190, 210-214, 230]. The nucleic acid substrate-specific biochemical properties of Dbp5 ATPase regulation defined here may have wide-ranging implications for Dbp5 functions in pre-ribosomal RNA export, translation, or R-loop metabolism. Furthermore, several DEAD-box proteins such as Dbp2 have been reported to bind diverse nucleic acid substrates (ncRNA and mRNA), including G-Quadraplexes, and have roles in R-loop biology [161, 234-240]. Given the high degree of conservation in the structure, function, and regulation of Dbp5 with other DEAD-box proteins [241, 242], the observations reported here raise the possibility that other DEAD-box proteins may also have substrate-specific regulation.

Overall, this study and other recent publications support a general function for both Dbp5 and Mex67 in RNA export [17, 19, 21, 63, 95, 179, 182, 186-189]. While these factors have been most well characterized in mRNA export, it is now important to reconsider Dbp5 and Mex67 as general RNA export factors, which raises questions about possible co-regulation of mRNA and non-coding RNA export pathways to fine tune gene expression. To this end, the *in vitro*, biochemical, and genetic properties of Dbp5 reported in this study will inform future structure-function and mechanistic characterization of a Dbp5 mediated, and Gle1 regulated, tRNA export pathway(s). For example, it will be important for future studies to address the composition of pathway specific export-competent tRNA-protein complexes and how Dbp5 contributes to changes in the architecture of such a complex as they move from nucleus to cytoplasm and back again.

3. 3. Figures


A
Autoradiography
untag DBP5
prA-DBP5
prA-dbp5L12A
prA-dbp5R423A
MW (kDa)
191
97
64
51
39
B
Background Control (Untag DBP5)
prA-DBP5
Total Reads=2433
Total Reads=12056
prA-dbp5L12A
prA-dbp5R423A
Total Reads=162054
Total Reads=207347
mRNA
tRNA
rRNA
snoRNA
snRNA
intergenic-granovskaia
antisense
intergenic
pseudogene
XUTs
antisense-granovskaia
antisense-Yassour
CUTs
SUTs
C
Silver Stain
untag DBP5
prA-DBP5
prA-dbp5L12A
prA-dbp5R423A
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D
Western Blot
untag DBP5
prA-DBP5
prA-dbp5L12A
prA-dbp5R423A
DBP5
RT-PCR
total RNA
untag DBP5
prA-DBP5
prA-dbp5L12A
prA-dbp5R423A
Cox1 mRNA
E
Unspliced tRNAIleUAU
Fold Enrichment
1000
100
10
1
DBP5
dbp5L12A
dbp5R423A

Figure 3-1: Dbp5 physically Interacts with pre-tRNA *in-vivo*

(A) Autoradiography performed to detect RNA isolated from Dbp5 CRAC following SDS PAGE. RNA can be detected migrating at the molecular weight of Dbp5 in Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} IPs, but not in nonspecific control IP.

(B) Dbp5 CRAC reveals broad interactions with mRNAs, rRNA and tRNAs. Dbp5-tRNA interactions are observed in Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} IPs, but do not appear to make up a large portion of reads in nonspecific control IP.

(C) Silver stains confirm Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} are free of contaminating proteins following formaldehyde crosslinking and IP. * Denotes Dbp5 and ** represent IgG eluted from dynabeads used for IP.

(D) Western blot analysis show enrichment of Dbp5, *dbp5*^{L12A}, and *dbp5*^{R423A} following IP. Additionally mitochondrial *Cox1* mRNA is not observed to be enriched in any IPs but is detectable in pre-IP input RNA.

(E) Formaldehyde crosslinking RNA-IPs show ~10-fold enrichment of pre-tRNA$^{Ile}_{UAU}$ in Dbp5 and *dbp5*^{R423A} IPs. This enrichment is elevated to ~100-fold in *dbp5*^{L12A}.

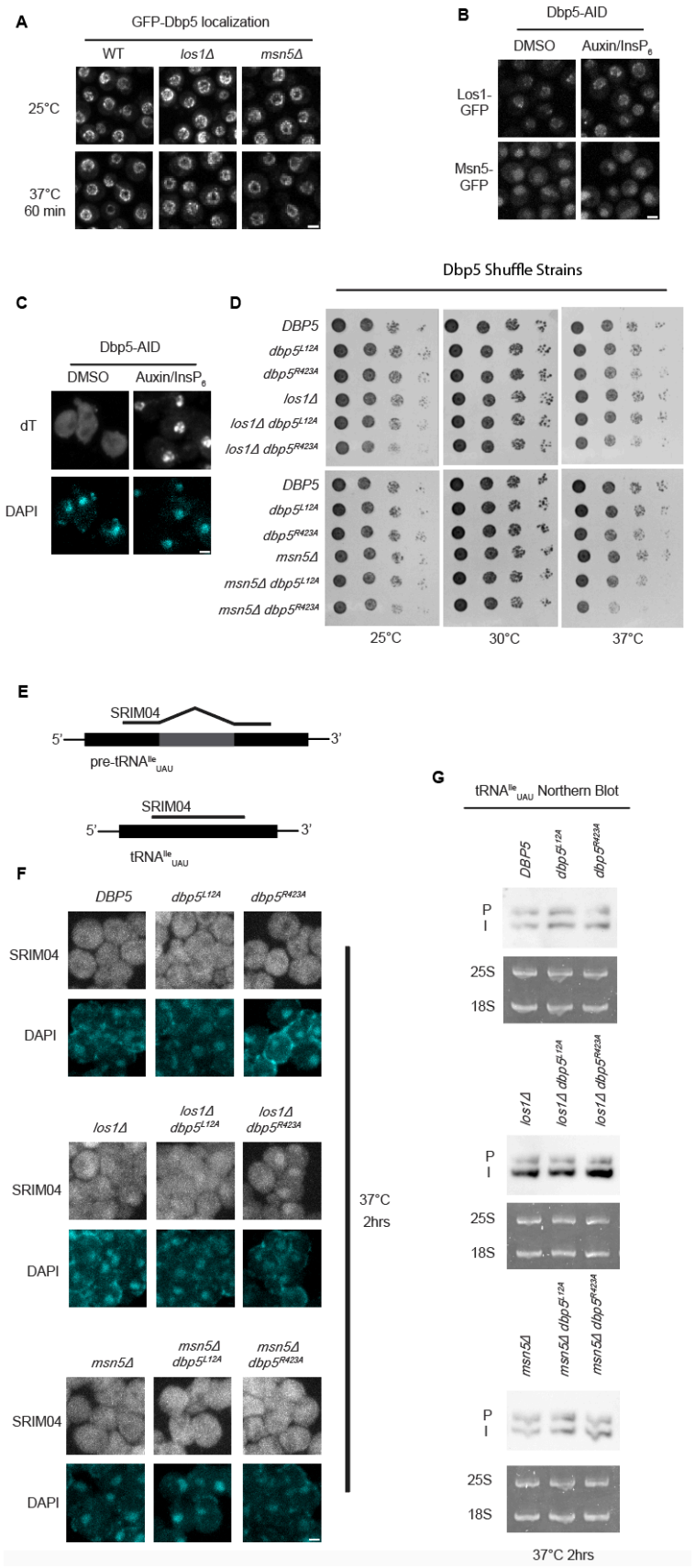
A
GFP-Dbp5 localization
WT
los1Δ
msn5Δ
25°C
37°C
60 min
B
Dbp5-AID
DMSO
Auxin/InsP6
Los1-GFP
Msn5-GFP
C
Dbp5-AID
DMSO
Auxin/InsP6
dT
DAPI
D
Dbp5 Shuffle Strains
DBP5
dbp5L12A
dbp5R423A
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
DBP5
dbp5L12A
dbp5R423A
msn5Δ
msn5Δ dbp5L12A
msn5Δ dbp5R423A
25°C
30°C
37°C
E
SRIM04
5'
3'
pre-tRNAIleUAU
SRIM04
5'
3'
tRNAIleUAU
F
DBP5
dbp5L12A
dbp5R423A
SRIM04
DAPI
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
SRIM04
DAPI
msn5Δ
msn5Δ dbp5L12A
msn5Δ dbp5R423A
SRIM04
DAPI
37°C
2hrs
G
tRNAIleUAU Northern Blot
DBP5
dbp5L12A
dbp5R423A
P
I
25S
18S
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
P
I
25S
18S
msn5Δ
msn5Δ dbp5L12A
msn5Δ dbp5R423A
P
I
25S
18S
37°C 2hrs

Figure 3-2: Analysis of Dbp5 and *los1Δ/msn5Δ* mutants

(A) Fluorescent images show GFP-Dbp5 remains enriched at the nuclear periphery in wild-type, *los1Δ,* and *msn5Δ* at 25°C and 37°C. Scale bar represents 2 µm.

(B) Los1-GFP and Msn5-GFP remains nucleoplasmic and associated with nuclear periphery in Dbp5-AID after treatment with DMSO or 500 mM Auxin and 10 mM $InsP_6$ for 90 minutes. Scale bar represents 2 µm.

(C) dT FISH confirms induction of mRNA export defect and Dbp5 loss of function after addition of 500 mM Auxin and 10 mM $InsP_6$ in Dbp5-AID strain for 90 minutes. Scale bar represents 2 µm.

(D) Spot assay for growth of strains containing untagged $dbp5^{L12A}$, $dbp5^{R423A}$ provided on CEN plasmid as sole copy of the protein when combined with either *los1Δ* or *msn5Δ* after two days at 25, 30, and 37°C on YPD.

(E) Diagrammatic representation of tRNAs targeted by SRIM04 probes used in (F).

(F) tRNA FISH targeting unspliced and spliced isoforms of $tRNA^{Ile}_{UAU}$ (SRIM04) in indicated strains after pre-culture to early log phase at 25°C and shift to 37°C for 2 hours. Scale bar represents 2 µm.

(G) Northern Blot analysis targeting precursor and mature isoforms of $tRNA^{Ile}_{UAU}$. Small RNAs were isolated from strains at mid log phase growth after pre-culture at 25°C and shift to 37°C for 2 hours.

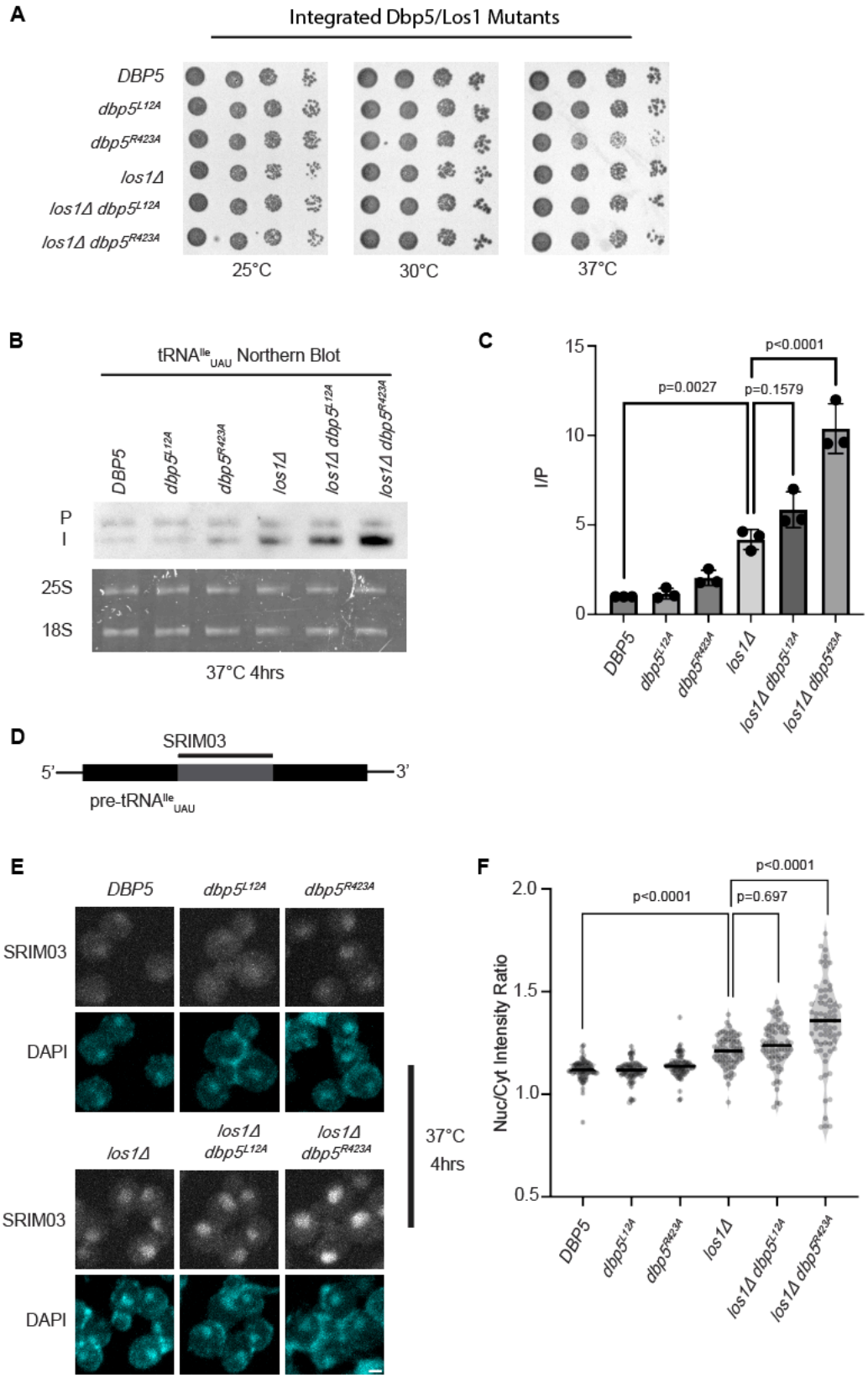
A
Integrated Dbp5/Los1 Mutants
DBP5
dbp5L12A
dbp5R423A
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
25°C
30°C
37°C
B
tRNAIleUAU Northern Blot
DBP5
dbp5L12A
dbp5R423A
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
P
I
25S
18S
37°C 4hrs
C
I/P
p=0.0027
p=0.1579
p<0.0001
D
SRIM03
5'
3'
pre-tRNAIleUAU
E
DBP5
dbp5L12A
dbp5R423A
SRIM03
DAPI
los1Δ
los1Δ dbp5L12A
los1Δ dbp5R423A
37°C
4hrs
F
Nuc/Cyt Intensity Ratio
p<0.0001
p=0.697
p<0.0001

Figure 3-3: Dbp5 functions parallel to Los1 in pre-tRNA export

(A) Spot assay for growth of strains containing untagged *dbp5*^{L12A}, *dbp5*^{R423A} and *los1Δ* integrated at genomic loci after two days of growth at 25, 30, and 37°C on YPD.

(B) Northern Blot analysis targeting precursor and mature isoforms of $tRNA^{Ile}_{UAU}$. Small RNAs were isolated from strains at mid log phase growth after pre-culture at 25°C and shift to 37°C for 4 hours.

(C) Quantification of Northern blot from (G). Ratio of signal from intron-containing end processed intermediates (I) vs 5' leader/3' trailer containing precursor (P) was calculated and presented relative to I/P ratio observed for WT. Error bars represent standard deviation and p-values calculated using one-way ANOVA.

(D) Diagrammatic representation of tRNAs targeted by SRIM03 probes used in (E).

(E) tRNA FISH targeting intron of $tRNA^{Ile}_{UAU}$ (SRIM03) in indicated strains after pre-culture to early log phase at 25°C and shift to 37°C for 4 hours. Scale bar represents 2 µm.

(F) Quantification of tRNA FISH from (E). Ratio of average nuclear to cytoplasmic pixel intensities were calculated across 3 independent replicate experiments and pooled for plotting. P-values were calculated using one-way ANOVA.

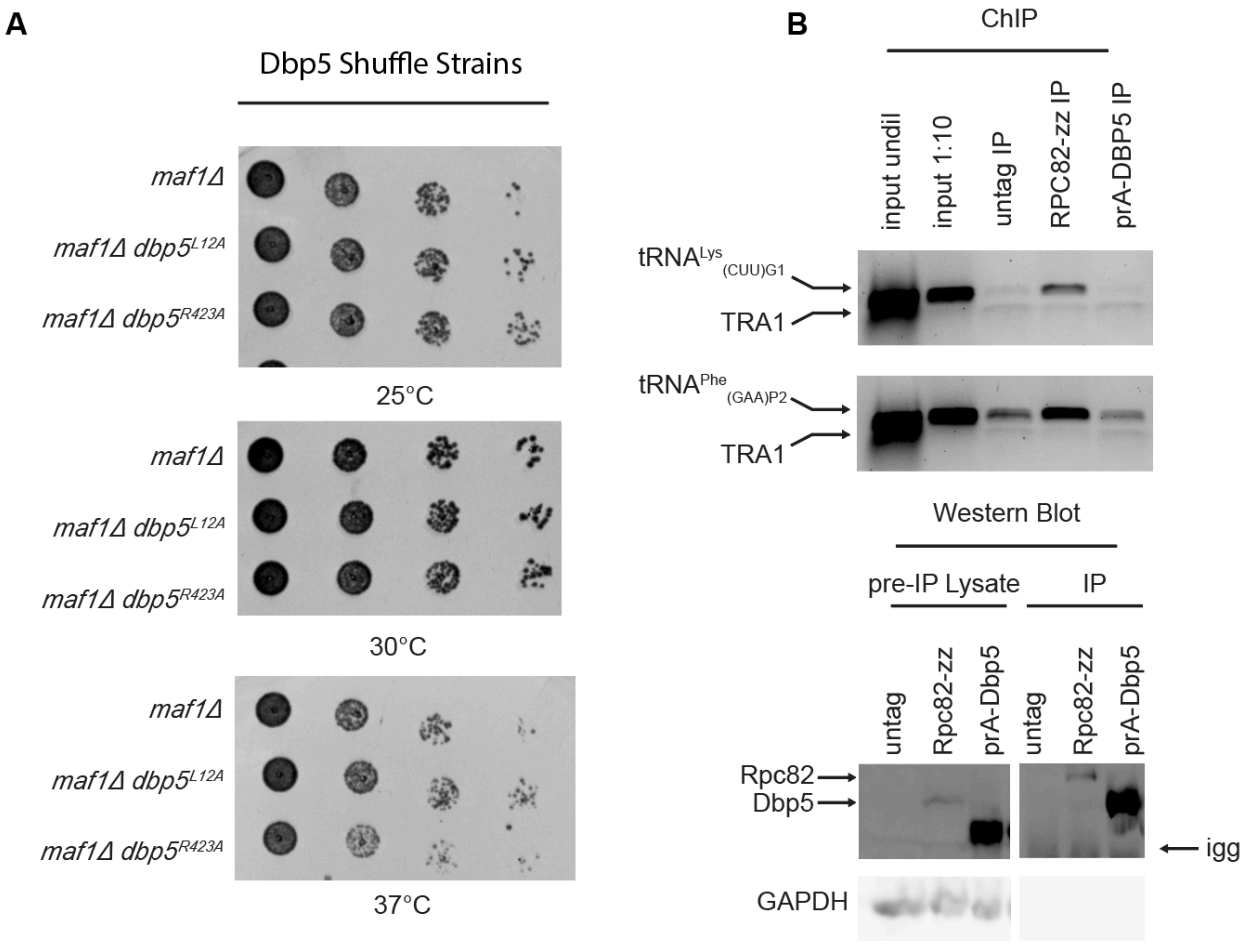
A
Dbp5 Shuffle Strains
maf1Δ
maf1Δ dbp5L12A
maf1Δ dbp5R423A
25°C
30°C
37°C
B
ChIP
input undil
input 1:10
untag IP
RPC82-zz IP
prA-DBP5 IP
tRNALys(CUU)G1
TRA1
tRNAPhe(GAA)P2
TRA1
Western Blot
pre-IP Lysate
IP
untag
Rpc82-zz
prA-Dbp5
Rpc82
Dbp5
igg
GAPDH
C
los1Δ
los1Δ dbp5R423A
thiolutin treated
thiolutin treated
time at 37°C:
t=0
t=30
t=60
t=120
t=120
P
I
25S
18S

Figure 3-4: Dbp5 and tRNA transcription

(A) Spot assay for growth of strains containing untagged *dbp5^{L12A}, dbp5^{R423A}* provided on CEN plasmid as sole copy of the protein when combined with *maf1Δ* after two days of growth at 25, 30, and 37°C on YPD.

(B) ChIP PCR targeting either tRNA$^{Lys}_{(CUU)G1}$ or tRNA$^{Phe}_{(GAA)P2}$. PCRs were multiplexed with nonspecific control target TRA1. IPs were performed on either lysate not expressing protein A tag, zz-Rpc82 or prA-Dbp5

(C) Western Blot analysis shows enrichment of Rpc82 and Dbp5 in IP fraction.

(D) Northern Blot analysis targeting precursor and mature isoforms of tRNA$^{Ile}_{UAU}$. RNA was isolated following shift to 37°C and treatment with thiolutin for indicated times.

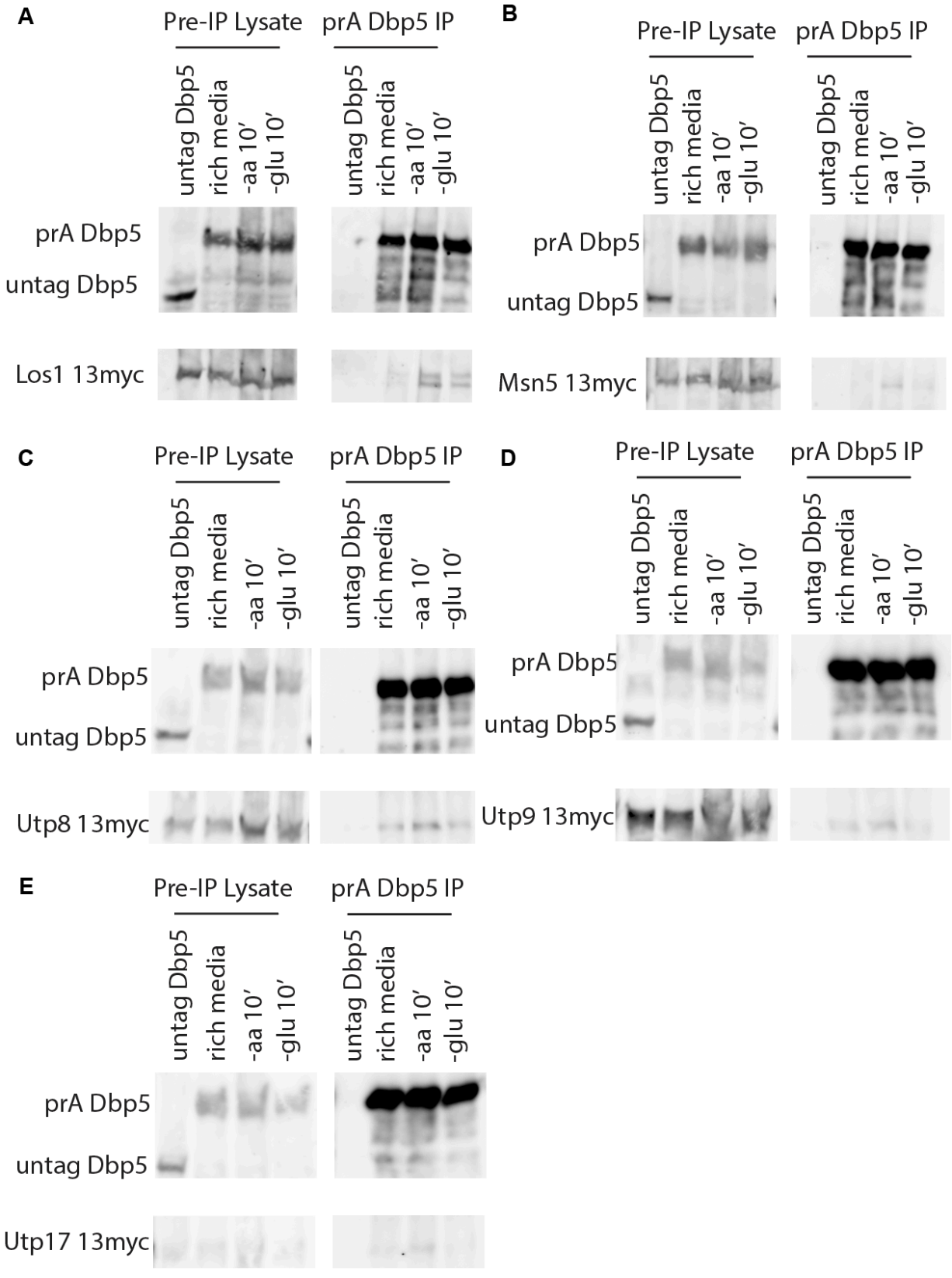
A
Pre-IP Lysate
prA Dbp5 IP
untag Dbp5
rich media
-aa 10′
-glu 10′
prA Dbp5
untag Dbp5
Los1 13myc
B
Pre-IP Lysate
prA Dbp5 IP
untag Dbp5
rich media
-aa 10′
-glu 10′
prA Dbp5
untag Dbp5
Msn5 13myc
C
Pre-IP Lysate
prA Dbp5 IP
untag Dbp5
rich media
-aa 10′
-glu 10′
prA Dbp5
untag Dbp5
Utp8 13myc
D
Pre-IP Lysate
prA Dbp5 IP
untag Dbp5
rich media
-aa 10′
-glu 10′
prA Dbp5
untag Dbp5
Utp9 13myc
E
Pre-IP Lysate
prA Dbp5 IP
untag Dbp5
rich media
-aa 10′
-glu 10′
prA Dbp5
untag Dbp5
Utp17 13myc

Figure 3-5: Dbp5 forms physical interactions with tRNA export factors

Co-IP experiments in which Dbp5 was isolated from yeast lysate, and Western Blot analysis was performed for (A) Los1, (B) Msn5, (C) Utp8, (D) Utp9, or (E) Utp17**.** IPs were conducted either after rich growth or after 10 minutes of amino acid or glucose starvation.

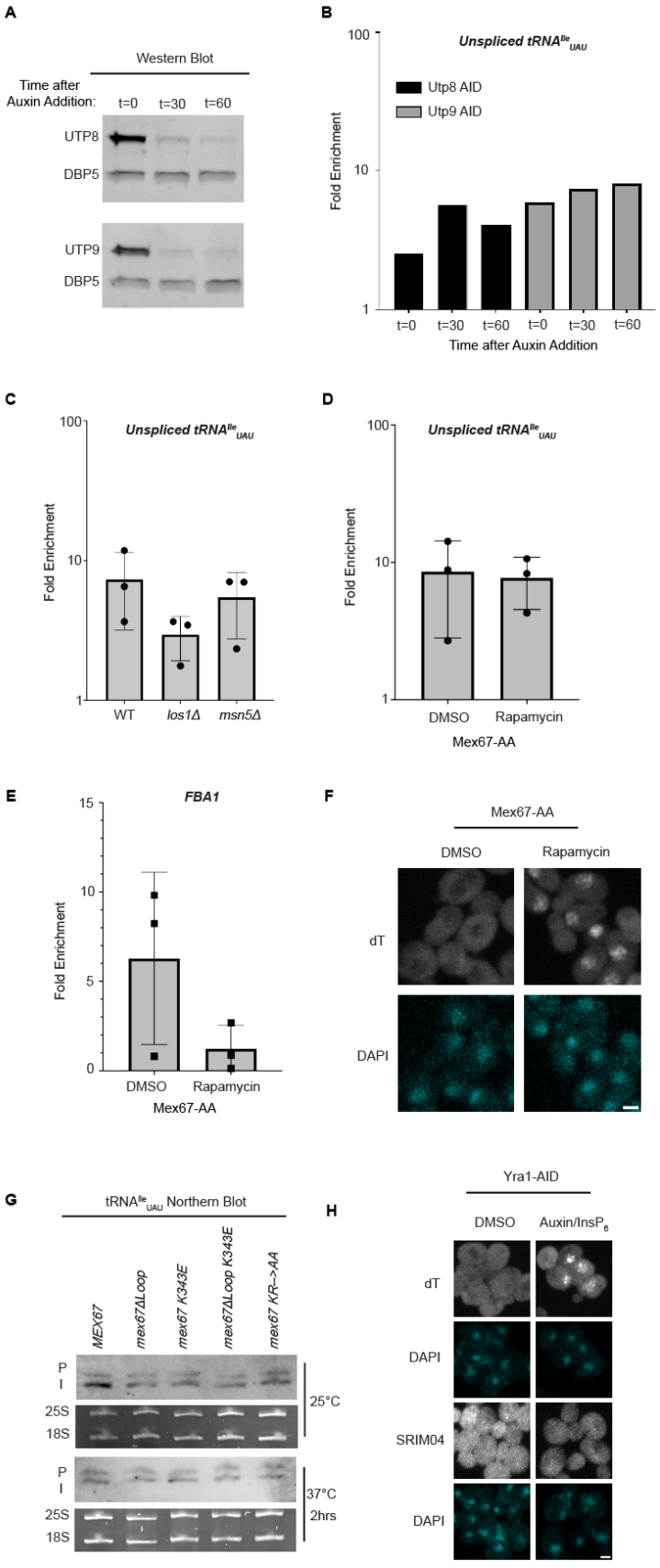
A
Western Blot
Time after Auxin Addition:
t=0
t=30
t=60
UTP8
DBP5
UTP9
DBP5
B
Unspliced tRNA$^{Ile}_{UAU}$
Utp8 AID
Utp9 AID
Fold Enrichment
100
10
1
t=0
t=30
t=60
t=0
t=30
t=60
Time after Auxin Addition
C
Unspliced tRNA$^{Ile}_{UAU}$
Fold Enrichment
100
10
1
WT
los1Δ
msn5Δ
D
Unspliced tRNA$^{Ile}_{UAU}$
Fold Enrichment
100
10
1
DMSO
Rapamycin
Mex67-AA
E
FBA1
Fold Enrichment
15
10
5
0
DMSO
Rapamycin
Mex67-AA
F
Mex67-AA
DMSO
Rapamycin
dT
DAPI
G
tRNA$^{Ile}_{UAU}$ Northern Blot
MEX67
mex67ΔLoop
mex67 K343E
mex67ΔLoop K343E
mex67 KR->AA
P
I
25S
18S
25°C
P
I
25S
18S
37°C
2hrs
H
Yra1-AID
DMSO
Auxin/InsP$_6$
dT
DAPI
SRIM04
DAPI

Figure 3-6: Utp8, Utp9, Los1 and Mex67 are not required for Dbp5 recruitment to pre-tRNA$^{Ile}_{UAU}$

(A) Western Blot analysis shows depletion of Utp8 and Utp9 following Auxin induced degradation at 30 and 60 minutes.

(B) Plots show relative fold enrichment of tRNA$^{Ile}_{UAU}$ following prA-Dbp5 RNA IP in a wild-type (WT) and Utp8 or Utp9 depletion for indicated time points. Abundance of target gene is normalized to abundance of the target transcript in input samples and represented as fold enrichment relative to RNA IP from an untagged control.

(C) Plots show relative fold enrichment of tRNA$^{Ile}_{UAU}$ following prA-Dbp5 RNA IP in a wild-type (WT), *los1Δ,* and *msn5Δ* strain background. Abundance of target gene is normalized to abundance of the target transcript in input samples and represented as fold enrichment relative to RNA IP from an untagged control.

(D) prA-Dbp5 RNA IP targeting tRNA$^{Ile}_{UAU}$ in Mex67-AA after either treatment with DMSO or 1 mg/ml rapamycin for 15 minutes.

(E) prA-Dbp5 RNA IP targeting FBA1 mRNA in Mex67-AA after either treatment with DMSO or 1 mg/ml rapamycin for 15 minutes.

(F) dT FISH confirming a mRNA export defect caused by loss of function of Mex67 following 15min incubation with 1 mg/ml rapamycin compared to a DMSO control. Scale bar 2 μm.

(G) Northern Blot analysis targeting precursor and mature isoforms of tRNA$^{Ile}_{UAU}$. RNA was isolated either after growth at 25°C or following shift to 37°C for 2hrs.

(H) tRNA FISH targeting unspliced and spliced isoforms of tRNA$^{Ile}_{UAU}$ (SRIM04) or dT FISH in Yra1 AID strains either without depletion or following 60min of depletion. Scale bar 2 μm.

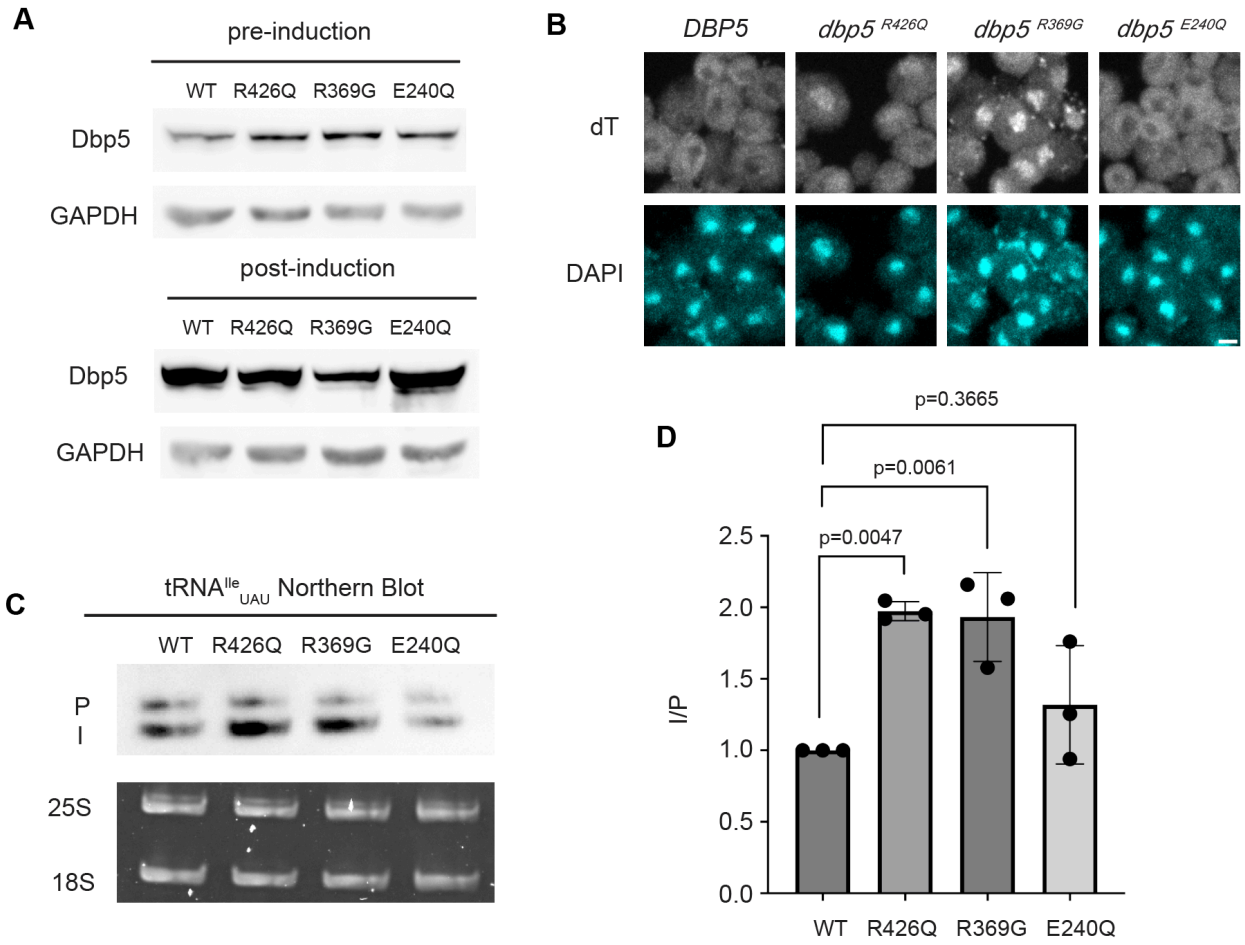
A
pre-induction
WT R426Q R369G E240Q
Dbp5
GAPDH
post-induction
WT R426Q R369G E240Q
Dbp5
GAPDH
B
DBP5
dbp5 R426Q
dbp5 R369G
dbp5 E240Q
dT
DAPI
C
tRNAIle UAU Northern Blot
WT R426Q R369G E240Q
P
I
25S
18S
D
p=0.3665
p=0.0061
p=0.0047
I/P
2.5
2.0
1.5
1.0
0.5
0.0
WT R426Q R369G E240Q

Figure 3-7: ATPase activity and Gle1 is required for Dbp5-mediated tRNA export in vivo

(A) Western blot with either mouse monoclonal anti-DBP5 or anti-GAPDH (loading control) antibody to detect overexpression of untagged Dbp5 and Dbp5 ATPase mutants. Strains were cultured in raffinose at 25°C to derepress pGAL promoter and expression was induced by shifting cultures into 2% galactose containing media for 6 hours. Expression was stopped by addition of glucose for 1 hour. Overexpression was observed over the level of wildtype Dbp5 that is expressed from the endogenous locus and is present in the pre-induction samples. Top and bottom panels are from the same blot and processed equally.

(B) dT FISH confirms previously reported mRNA export status phenotypes for Dbp5 ATPase mutants with *dbp5*^{R426Q} and *dbp5*^{R369G} showing a nuclear accumulation of poly(A)-RNA. Scale bar represents 2 µm.

(C) Northern blot analysis targeting precursor and mature isoforms of $tRNA^{Ile}_{UAU}$ from yeast strains overexpressing *DBP5*, *dbp5*^{R426Q}, *dbp5*^{R369G}, or *dbp5*^{E240Q}.

(D) Quantification of Northern blot from (C). Ratio of signal from intron-containing end processed intermediates (I) vs 5' leader/3' trailer containing precursor (P) was calculated and presented relative to I/P ratio observed for WT. Error bars represent standard deviation and p-values calculated using one-way ANOVA.

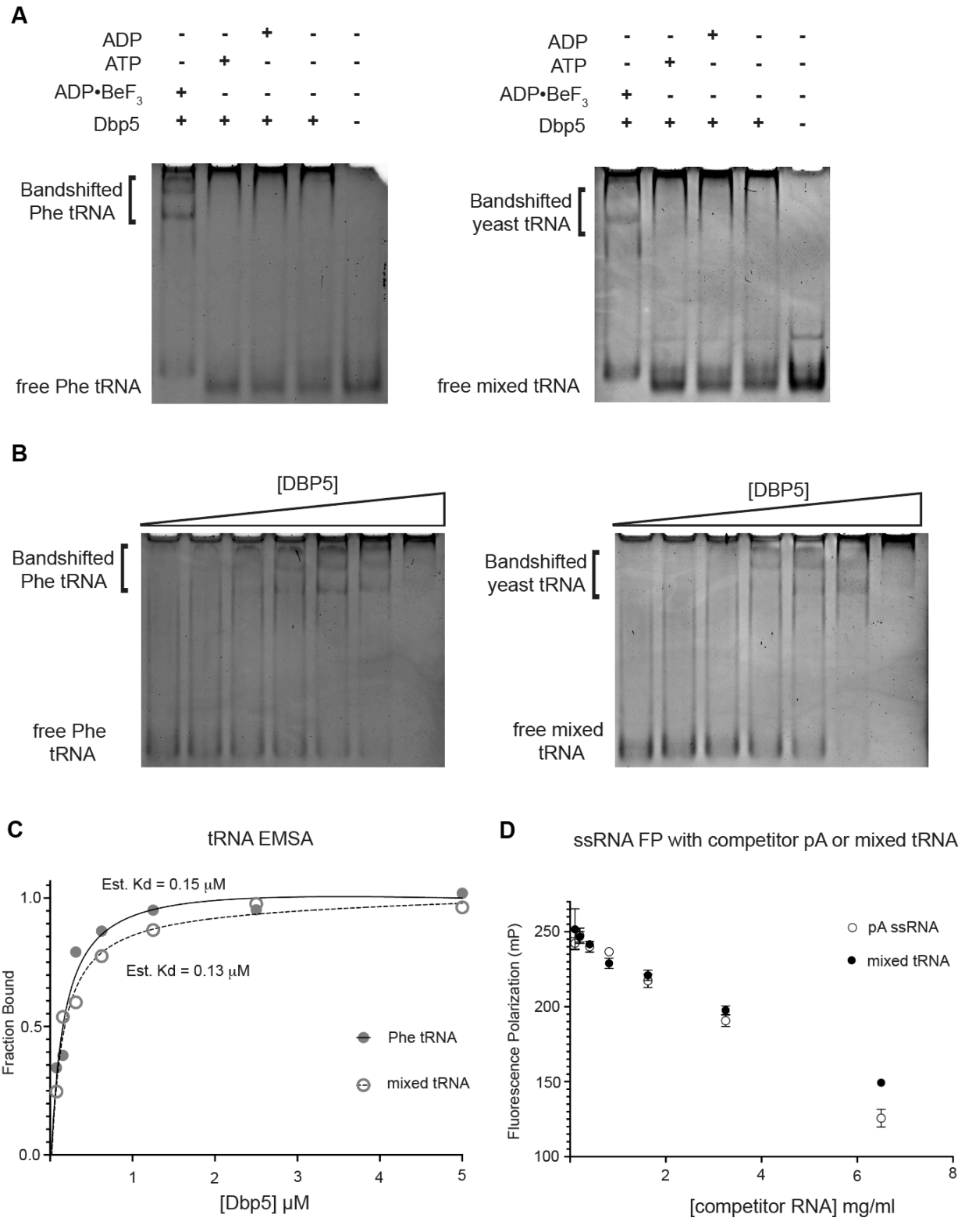
A
ADP
ATP
ADP•BeF3
Dbp5
Bandshifted Phe tRNA
free Phe tRNA
Bandshifted yeast tRNA
free mixed tRNA
B
[DBP5]
Bandshifted Phe tRNA
free Phe tRNA
Bandshifted yeast tRNA
free mixed tRNA
C
tRNA EMSA
Est. Kd = 0.15 μM
Est. Kd = 0.13 μM
Fraction Bound
Phe tRNA
mixed tRNA
[Dbp5] μM
D
ssRNA FP with competitor pA or mixed tRNA
Fluorescence Polarization (mP)
pA ssRNA
mixed tRNA
[competitor RNA] mg/ml

Figure 3-8: Dbp5 binds yeast tRNA in vitro

(A) Full length recombinant Dbp5 (fl-Dbp5) binds both mixed yeast tRNA and Phenylalanine (Phe) tRNA in the presence of the ATP mimetic ADP•BeF_3 by electrophoresis mobility shift assay (EMSA). RNA binding reaction was conducted in the presence of ADP•BeF_3, ATP, ADP, or no nucleotide and resolved on 6% native polyacrylamide gel at 70V. Reactions contained 2 µM fl-Dbp5, 1 mM nucleotide when present, 250 ng tRNA and binding buffer.

(B) EMSA experiments in which increasing concentrations of fl-Dbp5 was titrated into RNA binding reactions in the presence of 1 mM ADP•BeF_3, 250 ng mixed yeast tRNA or Phe tRNA, and binding buffer.

(C) Band intensities of free probe and band shifts were quantified, and bound fraction was calculated for each well of EMSAs in (B). One site binding model was fit to the data using GraphPad Prism to estimate Kd for both mixed yeast tRNA and Phe tRNA.

(D) Unlabeled pA ssRNA and mixed yeast tRNA compete with a 16nt fluorescein labeled ssRNA for fl-Dbp5 binding. Fluorescence polarization competition assays were performed by titrating increasing concentration of an unlabeled competitor, pA (open square) or mixed yeast tRNA (closed circle), in reactions containing 50 nM fluorescein labeled ssRNA, 2.5 mM ADP•BeF_3, 1 µM Dbp5 and buffer. Error bars represent standard deviation of three independent experiments.

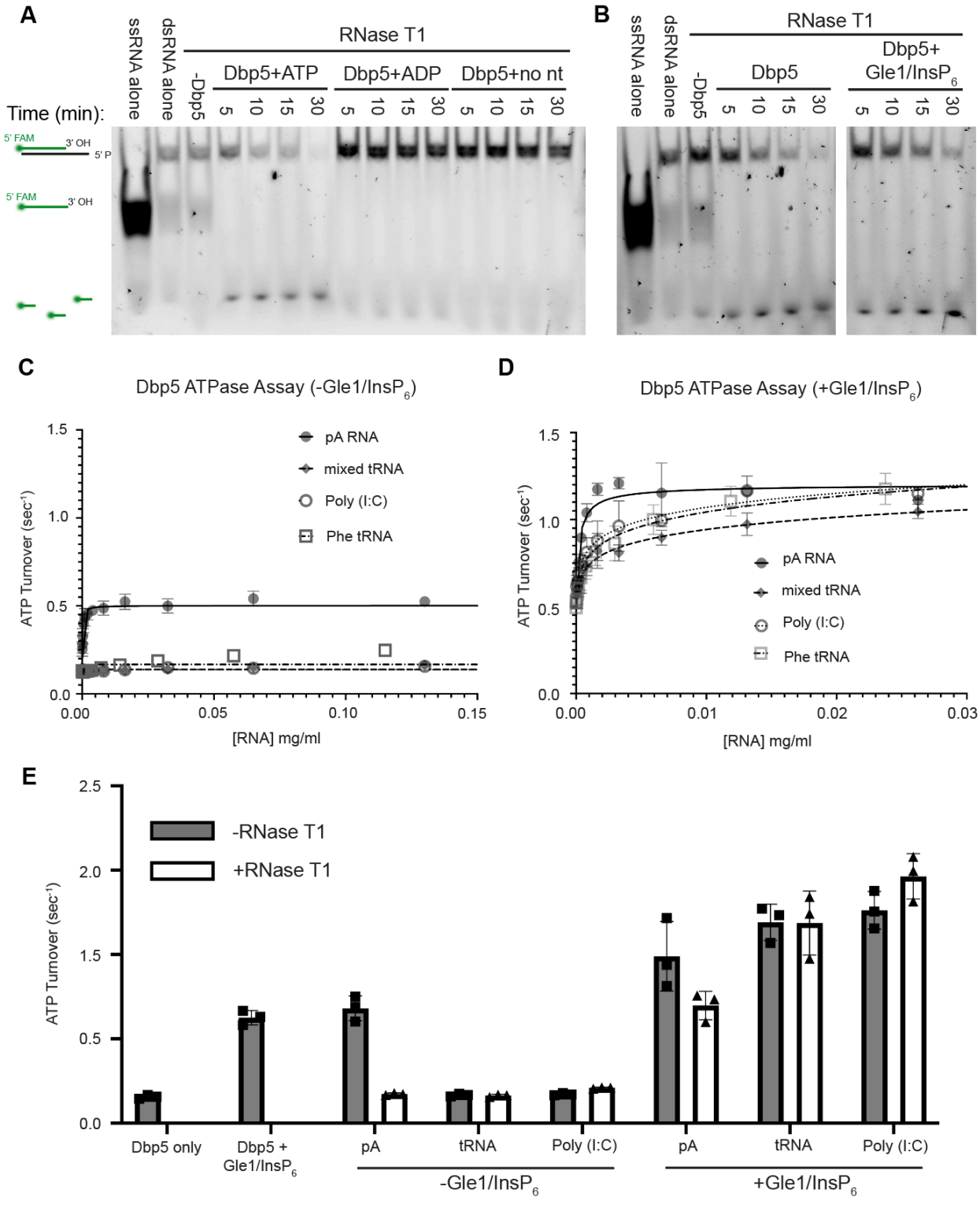
A
ssRNA alone
dsRNA alone
RNase T1
-Dbp5
Dbp5+ATP
Dbp5+ADP
Dbp5+no nt
Time (min):
5 10 15 30 5 10 15 30 5 10 15 30
5' FAM
3' OH
5' P
5' FAM
3' OH
B
ssRNA alone
dsRNA alone
RNase T1
-Dbp5
Dbp5
Dbp5+ Gle1/InsP6
5 10 15 30 5 10 15 30
C
Dbp5 ATPase Assay (-Gle1/InsP6)
ATP Turnover (sec-1)
[RNA] mg/ml
pA RNA
mixed tRNA
Poly (I:C)
Phe tRNA
D
Dbp5 ATPase Assay (+Gle1/InsP6)
ATP Turnover (sec-1)
[RNA] mg/ml
pA RNA
mixed tRNA
Poly (I:C)
Phe tRNA
E
-RNase T1
+RNase T1
ATP Turnover (sec-1)
Dbp5 only
Dbp5 + Gle1/InsP6
pA
tRNA
Poly (I:C)
pA
tRNA
Poly (I:C)
-Gle1/InsP6
+Gle1/InsP6

Figure 3-9: tRNA alone does not stimulate the Dbp5 ATPase cycle but can act synergistically with Gle1/$InsP_6$ to fully activate Dbp5

(A) Dbp5 dsRNA duplex unwinding assay performed in presence of ATP, ADP, or no nucleotide. Reactions were assembled and incubated with RNase T1 at 37°C for indicated times.

(B) Dbp5 dsRNA duplex unwinding assay performed in presence or absence of Gle1/$InsP_6$. Reactions were assembled and incubated with RNase T1 at 37°C for indicated times.

(C) ATPase activity of Dbp5 is stimulated by ssRNA (pA, closed square) but not mixed yeast tRNA (closed diamond), Phe tRNA (open square), or poly (I:C) dsRNA (open circle). Steady-state ATPase assays were conducted in the presence of 1 μM Dbp5, 2.5 mM ATP, and varying concentrations of RNA. Data was fit to Michaelis-Menten equation using GraphPad Prism. Error bars represent standard deviation of three independent experiments.

(D) Gle1/$InsP_6$ synergistically stimulates Dbp5 ATPase activity with mixed yeast tRNA (closed diamond), Phe tRNA (open square) and poly (I:C) dsRNA (open circle) substrates like ssRNA (pA). Steady-state ATPase assays were conducted in presence of 1 μM Dbp5, 2.5 mM ATP, 2 μM Gle1, 2 μM $InsP_6$, and varying concentration of RNA. Data was fit to an allosteric sigmoidal model in GraphPad Prism. Error bars represent standard deviation of three independent experiments.

(E) RNase T1 treatment of RNA for 2 hours at 37°C prior to ATPase assays confirms that the observed synergistic activation of Dbp5 ATPase activity by Gle1/$InsP_6$ and tRNA or dsRNA is not caused by low levels of contaminating ssRNA. Steady-state ATPase assays were conducted in presence of 1 μM Dbp5, 2.5 mM ATP, 0.2 mg/ml RNA and 2 μM Gle1/$InsP_6$ when indicated.

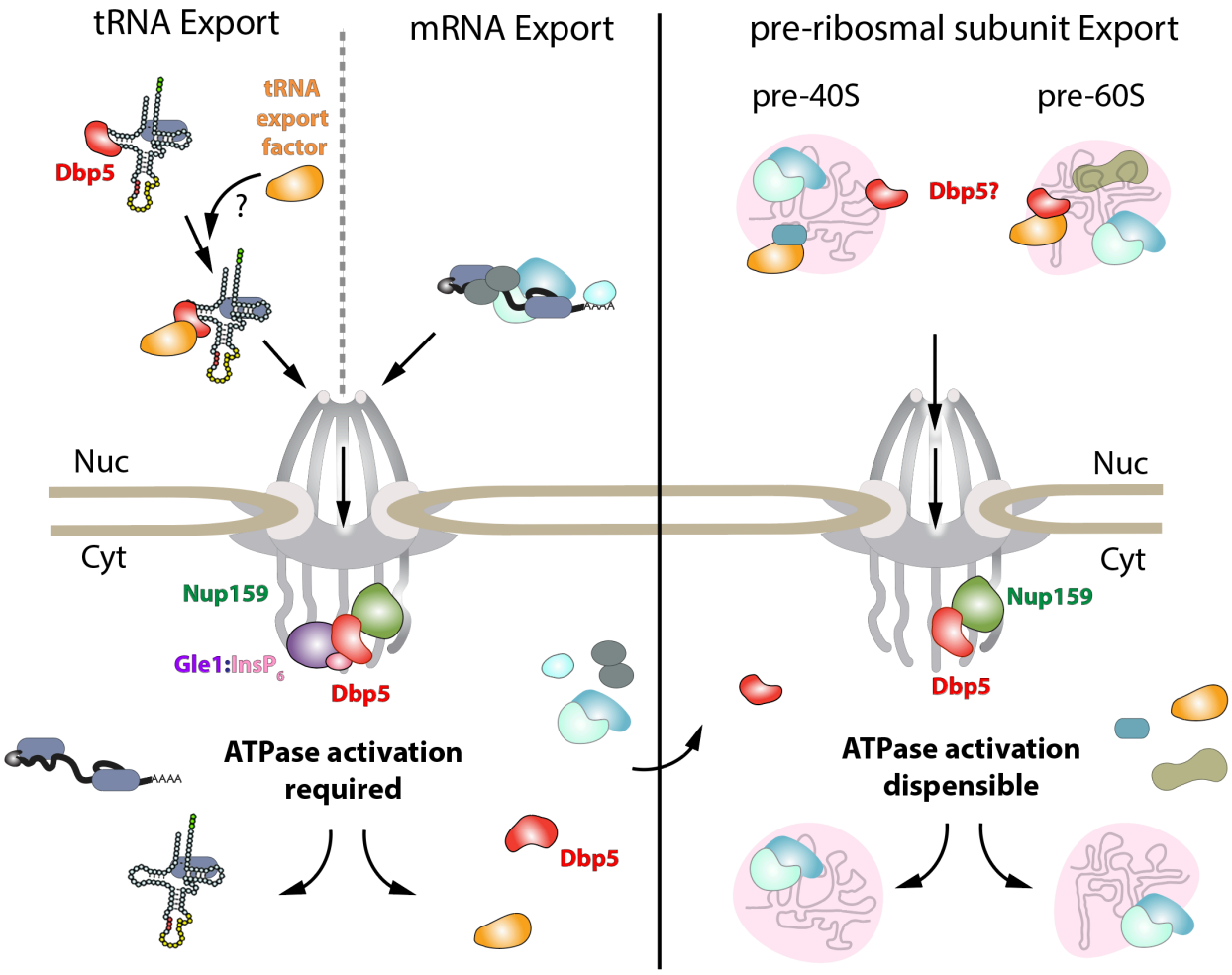
tRNA Export
mRNA Export
pre-ribosmal subunit Export
pre-40S
pre-60S
tRNA
export
factor
Dbp5
?
Dbp5?
Nuc
Cyt
Nup159
Gle1:InsP6
Dbp5
ATPase activation
required
Dbp5
ATPase activation
dispensible
Nup159
Dbp5
Nuc
Cyt

Figure 3-10: Model of Dbp5 function in mRNA, tRNA and pre-ribosomal subunit export

Data from this study support a model in which Dbp5-mediated export of both mRNA and tRNA require Gle1/$InsP_6$ activation to remodel RNPs at the cytoplasmic face of the Nuclear Pore Complex. For tRNA export, lack of RNA-mediated ATPase stimulation following RNA binding may lead to formation of tRNA bound intermediates that act as adapters for recruitment of yet to be identified transport factors. For mRNA export, it has been shown that Dbp5 function is limited to the nuclear periphery and Dbp5 does not form stable nuclear complexes with mRNA. Gle1/$InsP_6$ mediated activation of Dbp5 catalytic cycle and RNA release likely promotes recycling of export factors and Dbp5 to function in further rounds of export or other functions. In contrast, for Dbp5 mediated pre-ribosomal subunit export Dbp5 ATPase cycle and Gle1/$InsP_6$ stimulation are dispensable for transport. As such, RNA binding may promote export through an unknown mechanism and that Dbp5-mediated remodeling does not occur at NPCs, with factors such as Mex67 persisting on pre-ribosomal subunits following export to the cytoplasm.

Chapter 4: Concluding Remarks and Future Perspectives

Contrary to the prevailing view that ncRNA transport is a GTP driven process and distinct from the ATP driven mechanism of Dbp5 regulated mRNA export, my work reveals that Dbp5 and other mRNA export factors clearly and robustly participate in ncRNA biology. Here we show evidence for a physical interaction between Dbp5 and numerous ncRNAs and characterize a functional role for Dbp5-tRNA interactions *in-vivo*. Additionally, we report evidence that Dbp5 interacts with nucleolar proteins, snoRNAs, and snoRNA interacting proteins. Given that nucleolar localization of Dbp5 has been observed in $dbp5^{L12A}$ and there is nucleolar accumulation of tRNA in Dbp5 mutants, an early function of Dbp5 in the nucleus/nucleolus is likely. However, the details of this nuclear role remain elusive. In the context of tRNA biology, an attractive possibility is the ability of Dbp5 to serve as a clamp on pre-tRNAs within the nucleus/nucleolus. Mex67 is recruited to pre-tRNAs through an unknown mechanism and adapter. Furthermore, Mex67 is proposed to bind unprocessed 5' leader containing pre-tRNAs, suggesting tRNP association early in the tRNA life cycle [63]. Whether Dbp5 co-functions with Mex67 as an upstream adapter for this pathway or this represents yet another parallel mechanism will need to be further studied. More broadly, it is now clear that Dbp5 has functional roles in pre-ribosomal RNA export, tRNA export, and mRNA export.

Other nuclear DEAD-box proteins such as Dbp2 similarly have RNA-Interaction profiles with ncRNA that closely resemble Dbp5 [161, 239]. Previous reports of Dbp2-tRNA interactions and biochemical characterization of Dbp5-tRNA interactions raise questions about the broader implications of DEAD-box proteins as clamps and scaffolds mediated by cis-regulatory proteins

and RNA. We anticipate future experimentation in budding yeast will test and refine the models proposed here (Figure 1-3/3-10) by adding/removing interaction states using both *in vitro* (e.g. reaction rates and binding constants) and *in vivo* (e.g. mutational analysis and imaging) methods. For example, structural data is missing on the position of the NTD of Dbp5p when bound to nucleotide, RNA, and/or coregulators. This is functionally important and goes beyond auto-inhibition of the enzyme, as RNA-stimulation of Dbp5p requires the NTD [7]. Similarly, structural elucidation of the Dbp5-Gle1/$InsP_6$-tRNA complex will improve current understanding of how tRNA uniquely modulates the Dbp5 ATPase cycle.

The high level of conservation observed between NPC and RNA export machineries amongst yeast and metazoans further motivates continued investigation in both systems [145]; in addition to the properties of orthologs in other organisms. In mammals, two isoforms of Dbp5 exist (DDX19A and DDX19B) that share only 46% similarity in sequence identity to the yeast ortholog [166]. Elucidating how these differences relate to both the documented functions and regulation of yeast Dbp5p (e.g. spatial regulation, localization, and functions in ncRNA biology) is just beginning [128, 243]. This is particularly pertinent as recent work has linked functions of DDX19A and DDX19B to human health, especially within the context of viral infection [121, 244-246]. Core to addressing these questions will be a mechanistic understanding of Dbp5p regulation by co-regulators, the work that Dbp5p performs on an RNP, and the spatiotemporal dynamics of these activities. As a DBP, key to this will be understanding the interaction of Dbp5 with nucleotides, which is central to the ability of these enzymes to engage RNA. Similar to the Ran-GTP gradient, which regulate other nucleocytoplasmic transport events [15, 16, 247], we expect the sub-cellular distribution of different Dbp5 nucleotide states

is critical and likely regulated *in vivo* by co-regulators and possibly PTMs. This makes future characterization of Dbp5-nucleotide distributions and the identification of spatially restricted nucleotide exchange an important goal. Long term, we expect that a focus on these questions aimed at defining the core regulatory principles of Dbp5 will lead to a coherent model that unifies the diverse functions of Dbp5p in gene expression.

References

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