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Structure-Guided Mutational Analysis of a Yeast DEAD-box Protein Involved in Mitochondrial RNA Splicing

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Summary

DEAD-box proteins are RNA-dependent ATPase enzymes that have been implicated in nearly all aspects of RNA metabolism. Since many of these enzymes have been shown to possess common biochemical properties *in vitro*, including the ability to bind and hydrolyze ATP, bind nucleic acid, and promote helix unwinding, DEAD-box proteins are generally thought to modulate RNA structure *in vivo*. However, the extent to which these enzymatic properties are important for the *in vivo* functions of DEAD-box proteins remains unclear. To evaluate how these properties influence DEAD-box protein native function, we probe the importance of several highly conserved residues in the yeast DEAD-box protein, Mss116p, which is required for the splicing of all mitochondrial catalytic introns in *Saccharomyces cerevisiae*. Using a *MSS116* deletion strain, we have expressed plasmid-borne variants of *MSS116* containing substitutions in residues predicted to be important for extensive networks of interactions required for ATP hydrolysis and helix unwinding. We have analyzed the importance of these residues to the splicing functions of Mss116p *in vivo* and compared these results with the biochemical properties of recombinant proteins determined here and in previously published work. We observe that the efficiency by which an Mss116p variant catalyzes ATP hydrolysis correlates with facilitating mitochondrial splicing, while efficient helix unwinding appears to be insufficient for splicing. In addition, we show that each splicing-defective variant affects the splicing of structurally diverse introns to the same degree. Together, these observations suggest that the efficiency by which Mss116p catalyzes the hydrolysis of ATP is critical for all of its splicing functions *in vivo*. Given that ATP hydrolysis stimulates the recycling of DEAD-box proteins, these observations support a model in which enzyme turnover is a crucial factor in Mss116p splicing function. These results are discussed in the context of current models of Mss116p-facilitated splicing.

Keywords

DEAD-box proteins; helicase; catalytic introns; mitochondrial RNA processing; *Saccharomyces cerevisiae*

Introduction

DEAD-box proteins are ATPases that have been proposed to modulate RNA structure and/or RNA-protein interactions in nearly all aspects of RNA metabolism.^{1; 2} Members of this protein family share as many as eleven conserved sequence motifs within their catalytic core region,

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which span across two RecA-like domains.³ The N-terminal RecA-like domain contains the Q, I, Ia, GG, Ib, II, and III motifs, while the C-terminal RecA-like domain includes motifs IV, QxxR, V, and VI.³ Specific residues within these motifs are important for ATP binding, RNA binding, and forming *interdomain* interactions between the RecA-like domains that are required for duplex unwinding (see below).^{4; 5}

Structural and biochemical evidence have been used to propose a model in which DEAD-box proteins couple ATPase activity to RNA unwinding. In this model, binding of ATP and RNA promotes a conformational change that induces the RecA-like domains to form a “closed complex” that is strengthened through the creation of a network of *interdomain* interactions.^{5; 6; 7} The closure of these domains has been suggested to result in RNA “bending” and/or RNA “crimping” that causes local strand separation and subsequently leads to duplex unwinding.^{5; 8} Recent studies suggest that ATP binding is sufficient to cause helix destabilization, while ATP hydrolysis and/or inorganic phosphate release promotes the formation of an “open complex” and the release of the RNA from the protein.^{5; 6; 9; 10; 11} Therefore, the efficiency of ATP hydrolysis has been implicated in effective enzyme recycling.^{9; 10}

Despite these advances, very little is known about how DEAD-box proteins recognize their native substrates *in vivo* and to what extent the enzymatic properties established for these proteins *in vitro* are important for their function within the cell. Hence, the development of *in vitro* systems that recapitulate the activities of DEAD-box proteins in the context of their biological substrates, together with *in vivo* systems to easily monitor physiological phenotypes, are essential in efforts to elucidate their mechanisms of action.

A particularly useful model system to study DEAD-box proteins involves fungal mitochondrial splicing. Genetic analysis in yeast has revealed that the nuclear encoded DEAD-box protein, Mss116p, is required for the efficient splicing of all mitochondrial group I and group II introns *in vivo*, but how Mss116p facilitates splicing for each intron remains unclear.¹² Studies with recombinant Mss116p have shown that it can enhance the self-splicing efficiency of two yeast group II introns *in vitro*.^{13; 14} Based on these experiments, it was proposed that Mss116p functions to facilitate folding of an intron RNA into an active conformation either by acting as a general RNA chaperone to resolve misfolded structures or by stabilizing “on-pathway” intermediates during the folding pathway(s).^{13; 14; 15; 16; 17}

Recently, an additional *in vitro* splicing system was developed based on the yeast mitochondrial group I intron, $\alpha 5\beta$, from the cytochrome oxidase subunit I (*COX1*) gene.¹⁸ Two proteins, Mrs1p and Mss116p, were shown to facilitate the splicing of the $\alpha 5\beta$ intron.¹⁸ Mrs1p efficiently promoted the 1st step of $\alpha 5\beta$ splicing, while Mss116p specifically enhanced the efficiency of exon ligation via a mechanism that required ATP binding and hydrolysis. An analysis of Mss116p variants, which impaired its RNA-stimulated ATPase activity and/or reduced its ability to unwind a stable duplex *in vitro*, showed that the ability of Mss116p to efficiently hydrolyze ATP, rather than promote strand separation of a stable duplex, was important for its ability to facilitate $\alpha 5\beta$ exon ligation.

These results raised the question of whether efficient ATPase activity was sufficient for all of the splicing functions of Mss116p *in vivo*. To investigate this question, we deleted the *MSS116* gene from yeast and expressed variants of *MSS116* containing substitutions in residues predicted to form *interdomain* interactions that are required for coupling ATPase activity to helix unwinding. The biological effects of each substitution were then assayed *in vivo*. We show that, as in the case for $\alpha 5\beta$ splicing *in vitro*, the *in vivo* functions of Mss116p correlate with its ability to hydrolyze ATP. In addition, each splicing-defective variant of Mss116p affects the splicing of structurally diverse introns to the same degree. When compared to our

previous biochemical work, our results suggest that efficient unwinding is not sufficient for the *in vivo* functions of Mss116p. Rather, the efficiency by which Mss116p catalyzes the hydrolysis of ATP and, thus, the efficiency of enzyme turnover, appear to be the most critical for its *in vivo* splicing functions.

Results

Rationale for Selecting Sites of Structural Perturbation

To investigate the importance of residues implicated in forming *interdomain* contacts to the function of Mss116p *in vivo*, we created plasmid-borne variants of Mss116p. The substitutions were selected by reference to available crystal structures of the DEAD-box proteins Mss116p, Vasa, eIF4AIII, and DDX19.^{5; 8; 19; 20; 21; 22; 23} In all of these structures, the RecA-like domains were solved in a “closed” form and each protein bound a bent, single-stranded RNA and an ATP analog. In addition, the global conformation and the interactions involved in ATP binding, RNA binding, and *interdomain* interactions between the RecA-like domains were remarkably similar in all structures.

In this analysis, we made single alanine substitutions to residues in motifs III and QxxR, which have been implicated in forming *interdomain* interactions near the ATP and RNA binding sites, respectively. In this regard, a conserved threonine (T307_{Mss116p}) forms an *interdomain* interaction with a conserved histidine in motif VI, which is subsequently important for helping to form the ATP binding pocket (Fig. 1). In addition, a conserved glutamine residue (Q412_{Mss116p}) in the QxxR motif forms an *interdomain* interaction with a conserved arginine in motif Ia (Fig. 1). This interaction has been proposed to help form a continuous RNA binding surface and stabilize the bent form of the RNA strand (Fig. 1).

Effects of *Interdomain* Mutations

To study the importance of each residue in the function of Mss116p *in vivo*, we first created an *mss116* deletion strain (*mss116Δ*) by replacing the entire *MSS116* open reading frame with an antibiotic resistance gene (see Materials and Methods). We then transformed low copy plasmids containing each of the mutant genes, including 709 base pairs upstream and 322 base pairs downstream of the *MSS116* open reading frame, into *mss116Δ* and analyzed the ability of each strain to rescue respiratory growth when plated on glycerol. Since Mss116p is required for the processing of introns within mitochondrial encoded respiratory protein genes, the cells containing a deletion or a catalytically inactive form of Mss116p will grow slowly on a non-fermentable carbon source, such as glycerol, but the cells will retain the ability to utilize dextrose, a fermentable carbon source. Therefore, this glycerol growth test is a sensitive assay to monitor the biological affects of each mutation.

We compared the ability of each variant to rescue growth at 18, 24, 30, and 37 °C (Fig. 2). All strains grew equally well in the presence of dextrose at 30 and 37 °C, but growth of the null and K158A strains was reduced at 18 and 24 °C (Fig. 2, top panels). At 30 °C, wild-type Mss116p complemented the *mss116Δ* on glycerol as efficiently as the parent strain expressing the vector alone (*w.t.*-E.V.), whereas K158A, which inhibits ATP hydrolysis *in vitro*, did not rescue growth on glycerol, comparable to the null phenotype (Fig. 2, bottom panels).^{13; 18} The rescue of growth by the T307A and Q412A variants paralleled with their abilities to hydrolyze ATP *in vitro* (Fig. 2).¹⁸ T307A, which was previously measured as 6-fold less efficient at catalyzing ATP hydrolysis as the wild-type protein, grew substantially slower than the wild-type. In contrast, the Q412A variant, which was found to retain significant ATPase activity, rescued growth as efficiently as the wild-type protein. In addition, only the Q412A variant grew at 37 °C, similar to the wild-type strains. Lowering the temperature to 18 and 24 °C abolished the respiratory competence of the T307A variant, while Q412A showed a slow

growth phenotype only at 18 °C suggesting that it cannot fully complement the wild-type protein at very low temperatures. Together, these results suggest that the Q412A variant retains the ability to facilitate mitochondrial splicing, and the T307 residue is critical for at least one, or more, of the *in vivo* functions of Mss116p.

Dissection of Motif III

The *interdomain* interaction involving T307 appears to be important for the function of Mss116p *in vivo*, since an alanine substitution at this position impaired respiratory growth (Fig. 2). Motif III also contains a highly conserved serine residue (S305 in Mss116p; Fig. 1). Recent structural studies have revealed that both of these residues form *intradomain* interactions with the same conserved aspartic acid residue in motif II (Fig. 1).^{5; 8; 19; 20; 21; 22; 23} To delineate the significance of the *intradomain* and *interdomain* interactions involving motif III, we generated an additional variant of Mss116p by substituting an alanine at position S305. Growth on glycerol was then monitored at 18, 24, 30, and 37 °C (Fig. 3). Unlike the expression of the T307A variant, the S305A variant did not disrupt the function of Mss116p, since these cells grew as well as the wild-type strain at all temperatures examined. These observations signify that T307 is a central player within motif III and that a specific *interdomain* interaction involving this motif must be critical for the function of Mss116p.

Effects of *MSS116* Mutations on Mitochondrial Splicing Phenotypes

To analyze the effects of each substitution on the splicing of *al5β* and the overall processing of the *COX1* transcript *in vivo*, Northern hybridizations were performed on mitochondrial RNAs harvested from cells grown at 30 °C. To visualize precursor and mature forms of the *COX1* RNA, blots were hybridized with oligonucleotide probes complementary to the *COX1* exons, *aE5γ* and *aE6* (Figs. 4a and b). The abundance of each splicing isoform was quantified and standardized to the abundance of the *COX3* mRNA in each sample. A majority of the *COX1* RNA in the wild-type strain was fully processed, but at least 4 intermediate species were also observed (Fig. 4b, bands labeled C, D, E, and F). The abundance of exon ligation detected in each strain was similar using both exon probes (Fig. 4c).

Consistent with their poor growth on glycerol at all temperatures, the null strain (empty vector) and the strains expressing K158A and T307A primarily contained unprocessed, precursor RNAs that appeared to be very unstable, since precursor mRNAs and splicing intermediates were barely visible (Fig. 4b, see also Fig. 2). This observation was consistent with published literature, which documented that unprocessed precursor RNAs in yeast mitochondria are unstable.^{15; 24} Therefore, these mutants cause at least one intron to be inefficiently removed resulting in the precursor or intermediate(s) to be efficiently degraded.

Despite the observations that the strains expressing the S305A or Q412A variants grew as well as the wild-type strain on glycerol at 30 °C, the expression of these variants altered the fraction of *COX1* pre-RNA that underwent efficient processing (Fig. 4, see also Figs. 2 and 3). *COX1* exon ligation was reduced 30 and 40 % when S305A and Q412A were expressed, respectively, in comparison to the wild-type strain, and the distribution of the intermediate species was also altered. Species “F”, which was prominent in wild-type cells, was of lower abundance in the strains expressing the S305A or Q412A variants, and species “B”, which was not observed in the wild-type strain, accumulated (Fig. 4). The abundance of species “C, D, and E” also increased in both strains expressing the S305A or Q412A variants.

To examine the effects of each Mss116p variant on the splicing of the *al5β* intron, we employed a sensitive RT-PCR approach. Exon specific primers were used to PCR-amplify the product of *al5β* splicing for 26, 27, 28, or 34 cycles (Fig. 5a). Cycle 26 was the first cycle number in which ligated exons produced from *al5β* splicing could be perceptively detected in the

mss116Δ-E.V. strain, and the level of $\alpha 5\beta$ splicing was ~100-fold reduced compared to the *mss116Δ*-W.T. strain. At cycle 34, the relative fold difference in ligated exons between these strains was reduced to approximately 4. As such, the splicing patterns in cycle 26 best paralleled the patterns observed in the northern analysis and were chosen for quantitative analysis. $\alpha 5\beta$ exon ligation was barely detectable in the null strain and the strains expressing K158A and T307A, while the expression of S305A and Q412A efficiently facilitated a majority of $\alpha 5\beta$ exon ligation, as visualized by the cycle 26 amplicon (Figs. 5b and c).

Since Mss116p has also been shown to be required for the efficient splicing of all mitochondrial group I and group II introns in yeast, we expanded our analysis to examine the potential mutational effects on the splicing of the *COX1* group II intron, $\alpha 5\gamma$, and the *COB* introns, b11 (group II intron) and b13 (group I intron).¹² Since Mss116p has also been observed to be required for the translation of a subset of mitochondrial mRNAs in a yeast strain that lacks mitochondrial introns at temperatures lower than 30 °C, we primarily focused our investigation on the splicing of introns that do not require the translation of an intron-encoded maturase for efficient splicing *in vivo*.¹² The ω intron was not chosen for investigation because its *in vivo* splicing phenotype appeared much less sensitive to the deletion of *MSS116* than the other mitochondrial introns.¹²

Using exon specific primers to amplify each splicing product, the efficacy of $\alpha 5\gamma$, b11, and b13 splicing resembled the splicing phenotypes of the $\alpha 5\beta$ intron in the presence of each variant of Mss116p (Fig. 5). Splicing in the presence of the Q412A variant was similar to the wild-type, while the efficiency of splicing in the presence of the S305A variant was slightly lower (Fig. 5c). In contrast, very low amounts of splicing were detected in the presence of the K158A and T307A variants. In sum, each variant of Mss116p that compromised cell growth negatively affected intron processing to the same extent.

A Residue Implicated in RNA “Bending” is Important for the *In Vivo* Functions of *MSS116*

Based on the formation of *interdomain* interactions between the QxxR and Ia motifs, the conserved Q residue in the QxxR motif is predicted to help orient RNA binding residues near the most extreme RNA bending point (Fig. 1).^{5; 8; 22} Mutation of this residue could, in principle, disrupt the ability of the protein to stabilize the bent form of the RNA strand. In support of this prediction, the Q412A Mss116p variant did not efficiently unwind a stable duplex *in vitro*.¹⁸ However, this substitution only modestly altered the ability of Mss116p to facilitate the splicing of multiple introns *in vivo* (Figs. 4 and 5). Therefore, these results raised the possibility that the residues modeled to interact with the bent form of the RNA are not functionally relevant.

To determine if a residue that has been predicted to induce and/or stabilize the RNA bend in several DEAD-box proteins is important for the function of Mss116p *in vivo*, we made a single glutamic acid substitution at residue R245 in motif 1b. In the crystal structures of the Mss116p, Vasa, and eIF4AIII DEAD-box proteins, the corresponding residue was shown to be located at the RNA bending point and to recognize two phosphates and a 2'-OH group of the bent portion of the RNA strand (Fig. 6a).^{5; 8; 22} For Mss116p, the expression of R245E did not rescue respiratory growth or facilitate *COX1* and *COB* splicing (Fig. 6b-d). Therefore, these results establish the critical importance of residues that have been modeled to contact the bent RNA strand in the Mss116p/RNA structure.⁸

In vitro Activities of Mss116p Mutant Proteins

Previous investigations of wild-type Mss116p and the T307A and Q412A proteins revealed a strong correlation between each protein's ability to catalyze the hydrolysis of ATP and promote $\alpha 5\beta$ splicing *in vitro*, rather than promote strand separation.¹⁸ To measure the biochemical

activities of the R245E and S305A proteins, we cloned each gene into a bacterial expression vector and affinity purified the proteins from *E. coli*.¹⁸ The purification of these proteins, as well as those investigated previously, was optimized, since previously published (see Materials and Methods).¹⁸ As a result, the specific activity of each protein increased, but the relative activities of the mutant proteins to the wild-type remained the same (see Materials and Methods).

The ATPase activity of each protein was measured, using previously established conditions, in the presence of saturating concentrations of an *in vitro* transcript containing the *al5 β* intron RNA (Fig. 7).¹⁸ At 37 °C, wild-type Mss116p hydrolyzed ATP with a k_{cat} of $\sim 30 \text{ min}^{-1}$ and was characterized with a K_m of $\sim 167 \text{ }\mu\text{M}$ (Table 1; Fig. 7). The Q412A protein hydrolyzed ATP with a k_{cat} similar to the wild-type, while the T307A protein impaired ATP hydrolysis, and the K_m for ATP for both mutants were similar to the wild-type protein, as shown previously (Table 1; Fig. 7).¹⁸ In regards to the newly constructed variant proteins, the S305A protein had a similar K_m ($\sim 162 \text{ }\mu\text{M}$) and k_{cat} ($\sim 19.3 \text{ min}^{-1}$) for ATP hydrolysis as the wild-type protein (Table 1; Fig. 7). In contrast, the R245E substitution reduced the k_{cat} by 3.6-fold (Table 1; Fig. 7). Together, these results parallel with each protein's ability to facilitate intron splicing *in vivo*: the Q412A and S305A proteins efficiently catalyze ATP hydrolysis and facilitate intron splicing, while the T307A and R245E substitutions impair each protein's ability to hydrolyze ATP and promote *in vivo* splicing. In addition, the poor binding of ATP by R245E, as suggested by a 4-fold reduction in K_m , suggests that impaired ATP binding also results in impaired splicing (Table 1; Fig. 7).

To measure the efficiency of unwinding activity, we carried out a series of duplex unwinding assays in *al5 β* splicing conditions using a ^{32}P -labeled DNA oligo hybridized to an unlabeled RNA.¹⁸ Duplex unwinding follows biphasic kinetics using this substrate, as shown previously.¹⁸ The wild-type and S305A proteins were capable of unwinding the duplex in an ATP-dependent manner with similar efficiencies (Fig. 8; Table 2). R245E unwinding activity was similar to the wild-type in the absence of ATP, which correlates with its impairment in binding and hydrolyzing ATP (Tables 1 and 2; Figs. 7 and 8). These observations suggest that the biochemical activities of S305A are similar to the wild-type and, thus, it is not surprising that this variant supports intron splicing *in vivo*.

Under identical conditions, the T307A and Q412A proteins were both inefficient at unwinding the duplex relative to the wild-type, as described previously, but also repeated here for comparison (Fig. 8; Table 2).¹⁸ In this regard, the amplitude of the fast phase was significantly reduced (10- to 4- fold for T307A and Q412A, respectively) relative to the wild-type as was the rate (~ 2 -fold for each variant). In addition, the amplitude and rate of the slow phase were reduced by ~ 1.7 - and 2.7 - fold for both variants relative the wild-type. Taken together, these observations suggest that the efficiency of unwinding a stable duplex does not strongly correlate with promoting splicing *in vivo*.

Discussion

MSS116 is required for the splicing of all mitochondrial group I and group II introns in *S. cerevisiae*.¹² However, the mechanism by which Mss116p facilitates splicing for each intron remains unclear. To define the properties of Mss116p that are important for its roles in mitochondrial splicing, we analyzed the functional significance of several residues implicated in forming networks of *interdomain* interactions near the ATP and RNA binding sites. We created variants of Mss116p that were predicted to perturb these networks of interactions and investigated the roles of these residues in the *in vivo* splicing functions of Mss116p. When compared to our previous biochemical work, it appears that the efficiency by which an Mss116p

variant hydrolyzes ATP is the most important predictor of whether a given variant will promote efficient mitochondrial intron splicing.¹⁸

Dissection of Motif III: Mutations Do Not Phenocopy

Motif III (SAT) is the only conserved DEAD-box protein motif that does not appear to directly contact ATP or RNA.^{5; 8; 19; 20; 21; 22; 23} Historically, it has been suggested, from biochemical analysis, that motif III participates in coupling ATPase and unwinding activities. In this regard, a double mutation (SAT to AAA), in the eIF4A protein, inhibited duplex unwinding without decreasing ATPase activity.²⁵ In this study, we performed a thorough dissection of motif III to better understand the roles of the individual serine and threonine residues in the splicing function of Mss116p.

The expression of the S305A and T307A variants in the *mss116Δ* strain displayed contrasting phenotypes. While the expression of S305A permitted growth on glycerol at all temperatures examined, the expression of T307A severely inhibited mitochondrial function (Figs. 2 and 3). In support of these observations, expression of the T307A variant appeared to negatively affect the processing of the *COX1* and *COB* transcripts, while S305A expression resulted in efficient intron splicing for the *COX1* and *COB* pre-mRNAs *in vivo* (Figs. 4 and 5). Therefore, the serine and threonine substitutions did not phenocopy, and the T307 residue appeared to play a more critical role in the function of Mss116p. Similar phenotypic differences between serine and threonine variants in motif III have been documented for Has1p and Dbp4p, two yeast DEAD-box proteins involved in pre-rRNA processing.^{26; 27}

The molecular and biochemical phenotypes of these motif III variants provide a critical framework to understand the relative importance and function of individual molecular contacts observed in the crystal structures of other DEAD-box proteins. In this regard, motif III can be envisioned to possess two roles based on the structures of several DEAD-box proteins.^{5; 8; 19; 20; 21; 22; 23} First, motif III may help to stabilize the “closed” conformation when ATP and RNA are bound by forming *interdomain* interactions between the RecA-like domains. Second, motif III may help to organize a network of highly conserved residues that function to position the putative catalytic water in the ATP binding pocket, which is also formed upon the closure of the domains (Fig. 1).^{5; 8; 19; 20; 21; 22; 23} Specifically, the side chain of the Mss116p T307 residue is predicted to make an *interdomain* interaction with the side chain of H462, in motif VI (HRIGR), thus contributing to the network of interactions formed between the RecA-like domains (Fig. 1). In addition, both the S305 and T307 residues of Mss116p are predicted to make *intradomain* interactions with the highly conserved motif II residue, D270 (DEAD) (Fig. 1). In turn, D270 is projected to form an *interdomain* interaction with conserved motif V residue, R438 (RGMD), which is then anticipated to interact with the conserved E268 (DEAD) residue in motif II (Fig. 1). Together, the E268 (II) and H462 (VI) residues are proposed to interact with the presumptive catalytic water.

Based on these structural analyses, substitution of the conserved threonine residue would be predicted to be more detrimental than substitution of the serine residue, since it forms an *interdomain* contact, as well as an indirect contact with the putative catalytic water. Substitution of the serine residue would be predicted to disrupt the *intradomain* interaction with motif II, but this interaction may remain preserved, since the threonine residue also forms the same contact. Our observations are indeed consistent with these structural predictions.

The QxxR Motif and DEAD-box Protein Function

The crystal structure of the Vasa protein showed, for the first time, that a network of *interdomain* interactions was also formed near the RNA binding site. It was proposed that these interactions were important for correctly orienting the RecA-like domains in such a manner to

accurately position the RNA to induce strand bending, while having little or no effect on the geometry of the ATP binding pocket.⁵ Included within this network of contacts were the *interdomain* interactions between motifs QxxR and Ia, which have also been observed in other DEAD-box protein crystal structures, including Mss116p.^{5; 8; 22} In the Vasa protein, the conserved glutamine residue in motif QxxR was found to be critical for oogenesis in developing *Drosophila* egg chambers and coupling ATPase activity to unwinding activity in biochemical analyses.⁵ Hence, it was proposed that this residue was important for stabilizing the bent portion of the RNA. Thus far, this has been the only molecular and biochemical analysis of this highly conserved residue among DEAD-box proteins.

To further explore the importance of this conserved residue with regard to the function of DEAD-box proteins, we substituted an alanine at the corresponding position in Mss116p (Q412A, QxxR). In contrast to the Vasa protein, this substitution did not significantly disrupt the *in vivo* function of Mss116p, since cells expressing the Q412A variant rescued growth on glycerol and only moderately altered the processing of the *COX1* and *COB* pre-mRNAs (Figs. 2, 4, and 5). Yet, both “Q” variants of the Vasa and Mss116p proteins hydrolyzed ATP as well as the wild-type proteins, but were inefficient at unwinding model duplexes.^{5; 18}

Structurally, the side chain of Q412 in Mss116p is predicted to interact with the side chain of motif Ia residue, R190, which is then projected to contact two phosphates of the RNA backbone that include the phosphate at the bending point and the phosphate adjacent (5') to the bend (labeled as the U7 and U6 phosphates, respectively, in Fig. 1).^{5; 8; 22} Based on the structure of the Vasa protein, Mss116p Q412 is also predicted to interact with D191, a residue implicated in stabilizing the RNA binding residue, R415 (QxxR).³ Slight differences between the various crystal structures may reflect dynamic interactions involving the QxxR and Ia motifs.

Despite these structural predictions, efficient unwinding activity did not appear to be important for the function of Mss116p, which is in direct contrast with observations made for the Vasa protein.^{5; 18} What is the meaning of these contrasting scenarios? Assuming that some functions of Mss116p and Vasa require helix unwinding, it is important to note that *in vitro* unwinding efficiency of several DEAD-box proteins scales with duplex stability.^{9; 28; 29} It is possible that the “Q” variant of Mss116p may efficiently unwind helices of low stability *in vivo* and, therefore, would be expected to be phenotypically wild-type in the growth assays, if “unstable” helices are the native substrates of Mss116p. On the other hand, Vasa may function by disrupting an RNA-RNA interaction of higher stability than the corresponding substrates of Mss116p and, therefore, a “Q” variant of Vasa is disruptive *in vivo*. We suggest that this conserved glutamine residue may be a very important target for characterizing the structural nature of putative substrates of other DEAD-box proteins.

The *In Vivo* Functions of Mss116p Correlate with its Ability to Hydrolyze ATP

Most striking of our results was the ability of the Q412A (QxxR) variant to restore the respiratory competence of the *mss116Δ* strain and permit the majority of *COX1* and *COB* pre-mRNA processing, while the expression of the T307A (III) variant did not rescue the null phenotype. The Q412A recombinant protein was also as efficient at catalyzing the hydrolysis of ATP as the wild-type, whereas the T307A protein was impaired. As shown previously, as well as in Figure 8, the T307A and Q412A proteins were similarly poor in promoting strand separation of a stable model duplex *in vitro*.¹⁸ It was also clear that another protein variant with robust ATP binding and hydrolysis activity (S305A) supported splicing, while others with defective ATP binding and/or hydrolysis activity (R245E and K158A) did not. Taken together, our current and past observations support the idea that the efficiency of Mss116p ATPase activity appears to most accurately predict whether a given variant will promote efficient intron splicing *in vivo*. In addition, since Q412A supports splicing *in vivo*, it appears that the ability of Mss116p to unwind a stable duplex is not sufficient for facilitating splicing. It is important

to point out that distinguishing whether efficient ATPase activity or unwinding activity is important for Mss116p-facilitated splicing of the yeast mitochondrial $\alpha 5\beta$, $\alpha 5\gamma$, and b11 introns *in vitro* remains an on-going challenge.^{13; 14; 15; 16; 17; 18}

From previous analyses, Mss116p has been suggested to facilitate group II intron splicing in an ATP-dependent manner via two different mechanisms. First, Mss116p has been proposed to act as an ATP-dependent RNA chaperone to resolve misfolded structures in the $\alpha 5\gamma$ and b11 introns, by using its ability to unwind short, non-native helices in structured RNAs.^{14; 15} Alternatively, Mss116p has also been suggested to stimulate the splicing of the $\alpha 5\gamma$ intron by stabilizing “on-pathway” intermediates within the folding pathway through ATP-modulated RNA binding.^{13; 16}

Since these investigations were published, studies have suggested that ATP binding and ATP hydrolysis have separate roles in the unwinding activities of DEAD-box proteins *in vitro*. The binding of ATP and RNA has been proposed to be sufficient to induce local strand separation, while the hydrolysis of ATP promotes enzyme turnover.^{9; 10} However, it was suggested that each cycle of ATP binding and hydrolysis does not always lead to successful strand separation.⁹ Therefore, the substrate may need to encounter an enzyme multiple times before the strands dissociate, which would require many rounds of ATP binding and hydrolysis.⁹ With this in mind, impairment in the abilities of DEAD-box proteins to hydrolyze ATP could lead to detrimental effects on cellular functions. Below, we describe these consequences in the context of both proposed models for the functions of Mss116p.

In one model, Mss116p has been suggested to act as an ATP-dependent RNA chaperone to resolve kinetically trapped RNA substrates by binding non-specifically and inducing local strand separation (Fig. 9a).^{14; 15} Since all binding events may not result in complete separation, one can envision the need for multiple encounters with any given intron (Fig. 9a). In this regard, higher efficiency of chaperone binding and release could result in a larger population of intron RNAs in the destabilized state, thereby providing more opportunities for the intron RNA to successfully re-fold into a functional, native state. A decrease in the efficiency of Mss116p to hydrolyze ATP, and subsequently to turnover, may reduce the population of destabilized RNAs that may potentially reach the native state.

Inefficient ATP hydrolysis may also be detrimental when considered in the context of the second model of Mss116p function. In this regard, Mss116p has been proposed to capture a short lived, single-stranded RNA folding intermediate through ATP-dependent RNA binding and promote the ordered folding of the remaining domains of the $\alpha 5\gamma$ intron RNA (Fig. 9b).^{13; 16} In this scenario, the binding of Mss116p and its release of the RNA substrate would need to be temporally controlled to accurately facilitate splicing (Fig. 9b). If Mss116p is slow to hydrolyze ATP and release the intermediate, the propensity of the RNA to mis-fold into a stable, off-pathway intermediate could increase, thus decreasing the efficiency of pre-mRNA splicing. In either model, inefficient enzyme turnover by Mss116p could lead to inefficient intron splicing and disruption of cellular function.

It seems likely that Mss116p may facilitate the splicing of its 13 different mitochondrial substrates via different mechanisms. Despite these potential differences, the ability of Mss116p to efficiently catalyze the hydrolysis of ATP is of central importance in all cases. Further studies using *in vitro* model systems will provide new insights into the mechanisms by which Mss116p functions in the context of its native substrates. In addition, these systems coupled with *in vivo* assays will provide a framework to understand the relative importance of enzymatic properties, such as ATP hydrolysis and unwinding activities, to the precise function of Mss116p for individual splicing events. Results from these studies will likely be broadly applicable to

understanding the roles of additional DEAD-box proteins in RNA splicing, as well as in other areas of RNA metabolism.

Materials and Methods

Yeast strains, expression plasmids, and growth conditions

In this report, the *Saccharomyces cerevisiae* wild-type (*w.t.*) strain is a derivative of the BY4741 strain, as described previously.³⁰ To generate the *mss116Δ* strain, the entire *MSS116* open reading frame was replaced with a *kan^r* cassette, using primers 07B05 and 07B06 and the methods previously described (see Supplementary Table 1).³⁰ PCR amplification, using primers 07A09 and 07A10 (see below; Supplementary Table 1), confirmed the insertion of the *kan^r* cassette at the correct genomic location.

To generate the wild-type (W.T.) rescue plasmid, the 07A09 and 07A10 primers (Supplementary Table 1) were used to amplify genomic sequence encompassing 709 bp of sequence upstream of the *MSS116* start codon, the entire 1995 bp *MSS116* open reading frame, and 322 bp of sequence downstream of the *MSS116* termination codon from the parent BY4741 strain. This PCR product and linearized CEN6 pRS415 plasmid (#87520, ATCC®) were transformed into the *mss116Δ* strain and the rescue plasmid was generated via homologous recombination in yeast. Positive transformants were selected as described.³⁰ Point mutations in *MSS116* were constructed by site-directed PCR mutagenesis³¹ using the appropriate mutagenic primers together with the 07A09 and 07A10 primers, and the plasmids generated as formerly described.³⁰

To assay the biological effects of each *MSS116* mutation, CEN6 pRS415 plasmids containing wild-type or mutant *MSS116* were transformed into the *w.t.* or *mss116Δ* strains using 0.1 μg and 1.0 μg of plasmid DNA, respectively, selected as described.³⁰ Transformants were selected on SCAD/-Leu and SCG/-Leu medium supplemented with 0.01 % adenine (SCAD/-Leu or SCAG/-Leu).³⁰ For growth analysis, cells were grown in liquid SCAD/-Leu, diluted to an OD₆₀₀ of 1, serially diluted (1/10, 1/100, 1/1000), and 5 μL of each dilution was spotted onto SCAD/-Leu and SCAG/-Leu plates.

Mitochondrial RNA isolation

Mitochondrial RNA (mtRNA) was isolated from *mss116Δ* strains transformed with plasmids containing wild-type or mutant *MSS116*. Cells were grown in SCAD/-Leu media (600 mL) at 30 °C to an OD₆₀₀ of 1.5, and the mtRNA was purified exactly as described.³⁰

Northern blots

Gene specific primers used for Northern analysis are described in Supplementary Table 2. In brief, primers 06B03 and 04A03 were used to detect the *aE5γ* and *aE6* exons, respectively. The *COX3* transcript was detected using the 03J07 primer. Radio-labeling of gene specific primers and northern analysis were completed as described.³⁰

RT-PCRs

The synthesis of cDNAs and RT-PCR were completed nearly as described.³⁰ In sum, SuperScript™ III Reverse Transcriptase (Invitrogen) and the recommended protocol were used to synthesize cDNAs from 50 ng/μL of each mtRNA, but with the following two exceptions: The RNA was incubated with primers and dNTPs at 70 °C for 5 min and 10 U of RNase Out™ Recombinant RNase Inhibitor (Invitrogen) was added to each reaction. For the synthesis of *COB* cDNAs, the reactions were incubated at 37 °C for 2 min prior to incubation at 55 °C for 60 min. Five units of RNase H (New England Biolabs) were used to digest the RNA complementary to the cDNA. Each cDNA was then diluted 1:20 and 2 μL was used as a

template for PCR. Pre-optimized Diamond Mix (Bioline, Taunton, MA) was used to amplify each splicing product in the presence of an additional 5 mM MgCl₂ and 0.4 μM primer sets (final concentrations in a 50 μL reaction volume). Each reaction was denatured at 94 °C for 2 min, followed by 26 to 34 cycles at 94 °C for 15 sec / 50 °C for 15 sec / 72 °C for 1 min in a TC-412 PCR machine (Techne, Inc., Burlington, NJ). Amplification of the *al5γ* splicing was done using an annealing temperature of 65 °C. Reaction products were separated on a 2.5 % agarose –1× TAE gel, detected using ethidium bromide staining, visualized with the ChemiGenius² system and GeneSnap software (Syngene, Frederick, MD), and quantified using ImageQuant 5.2 software (Molecular Dynamics).

The gene specific primers used to generate each cDNA and to amplify the products of splicing are described in Supplementary Table 2. For *COX1* splicing, *al5β* exon ligation yielded a 160 bp product using primers 03F03 and 06B03 and *al5γ* exon ligation yielded a 255 bp product using primers 08J06 and 04A03. For *COB* splicing, *b11* exon ligation yielded a 374 bp product using primers 08J07 and 08J08 and *b13* exon ligation yielded a 147 bp product using primers 03B08 and 03C08. *COX3* amplification yielded 335 bp product using primers 03I07 and 03J07. The reverse primer from each primer set was used to generate the corresponding cDNA for PCR analysis. The abundance of ligated exons was normalized to the amplification of the *COX3* transcript.

Recombinant proteins and *in vitro* assays

Recombinant Mss116p was cloned, expressed, and purified essentially as described,¹⁸ except with the following changes. During purification, the column resin was washed with 100 mL of TN buffer supplemented with 5 mM imidazole, followed by a second wash with 30 mL of TN supplemented with 30 mM imidazole, followed by a third wash with 100 mL of TN supplemented with 5 mM imidazole. Finally, the Mss116p protein was eluted with 6 mL (1 mL fractions) of TN supplemented with 200 mM imidazole. The combination of these additional wash steps and a reduction in the amount of protein that became insoluble during dialysis enhanced the specific activity of each protein ~5-7 fold, but the relative activity of each mutant to the wild-type remained the same (see Table 1).

The procedures for the ATPase assays are identical to those described.¹⁸ The v_o for R245E measured at 1 mM ATP was within error of the rate measured at 0.5 mM and, therefore, ATP was saturating. Unwinding assays were also performed and analyzed as described.¹⁸

Crystal structure modeling and analysis

Structural figures were prepared using the SwissPdb Viewer, <http://www.expasy.org/spdbv/>.³² The structures were prepared using the coordinates for the Mss116p protein bound to a polyU-RNA and the ATP analog, AMP-PNP, which are available from the Protein Data Bank with accession code, **3IFX** (Del Campo 2009).⁸ The interactions shown in Figs.1 and 6 were highly conserved among the structures of additional DEAD proteins, including *Drosophila* Vasa solved in complex with a polyU-RNA and AMP-PNP (PDB ID: **2DB3**),⁵ human eIF4AIII solved in complex with a polyU-RNA and AMP-PNP (PDB IDs: **2J0S** and **2HYI**),^{19; 20} human eIF4AIII solved in complex with a polyU-RNA and ADP-AIF₃ (PDB ID: **3EX7**, complex 1),²² human DDX19 solved in complex with a polyU-RNA and AMP-PNP (PDB IDs: **3FHT** and **3G0H**),^{21; 23} and yeast Mss116p solved in complex with a polyU-RNA and ADP-BeF₃⁻ or ADP-AIF₃⁻ (PDB IDs: **3I61** and **3I62**).⁸ The overall structures of Dbp5/DDX19 structures were very similar to the structures of these other DEAD-box proteins, despite differences in the conservation of the motif sequences.^{21; 23}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Networks of conserved *interdomain* interactions in “closed” conformation of Mss116p and additional DEAD-box proteins. (a) Schematic of Mss116p conserved sequence motifs. Motifs Q through III are located in the N-terminal domain, and motifs IV through VI are located in the C-terminal domain. The motifs that form interdomain interactions are colored, and the dotted lines represent interactions between motifs.^{5; 8; 19; 20; 21; 22; 23} Residues in “bold” are the specific residues mutated in this investigation (K158A, R245E, S305A, T307A, Q412A). (b) Structural representations of interactions involving motifs III and QxxR. The green dotted lines represent hydrogen bonds between residues. The crystal structure of the Mss116p protein bound to a polyU-RNA and AMP-PNP was used to create these figures (see Materials and Methods).⁸ These interactions have been predicted from additional DEAD-box protein crystal structures.^{5; 8; 19; 20; 21; 22; 23} For clarity the interaction between the backbone of T307 and the side chain of S305 is not shown, as well as multiple interactions involving the backbone of Q412.

Fig. 2.

Growth phenotypes of wild-type and mutant derivatives of Mss116p. CEN6 plasmids containing wild-type (W.T.) and mutant derivatives of Mss116p (K158A, T307A, Q412A) or the pRS415 vector control (E.V.) were transformed into the wild-type (*w.t.*) or *mss116Δ* strains. Cells were grown in liquid, dextrose-containing medium at 30 °C, serially diluted, spotted on dextrose- or glycerol-containing medium, and incubated at the indicated temperatures.

Fig. 3.

Growth phenotypes of wild-type and mutant derivatives of Mss116p. CEN6 plasmids containing wild-type (W.T.) and mutant derivatives of Mss116p (S305A, T307A) or the pRS415 vector control (E.V.) were transformed into the wild-type (*w.t.*) or *mss116Δ* strains. Cells were grown in liquid, dextrose-containing medium at 30 °C, serially diluted, spotted on dextrose- or glycerol-containing medium, and incubated at the indicated temperatures.

Fig. 4. Splicing phenotypes of *mss116Δ* strains expressing variants of Mss116p assessed by Northern blot analysis. (a) Schematic of the *COX1* pre-mRNA transcript containing eight exons and seven introns, with nomenclature designated as in Valencik et. al., 1989.³³ G.I, group I introns; G.II, group II introns. (b) Representative northern blots of total mitochondrial RNA from *mss116Δ* strains harboring mutant derivatives of Mss116p. RNA was harvested from cells grown in dextrose-containing media at 30 °C. Blots were hybridized with 5'-³²P-labeled DNA oligonucleotides complementary to the *COX1* exons aE5γ and aE6, or the intron-less *COX3* transcript, which served as the loading control. Different splicing isoforms are indicated by letters A-F. Fully processed pre-mRNAs are marked as LE for ligated exons. (c) Histogram of exon ligation in each mutant strain relative to exon ligation in the wild-type strain, as detected using both the aE5γ and aE6 exon probes. All data was normalized to the abundance of the *COX3* RNA. The data using the aE5γ probe are the average from two independent experiments and the error bars represent the range of the values divided by two. Exon ligation in the strains expressing the E.V., K158A, and T307A variants was very low in abundance or essentially zero and may not be seen on the histogram.

Fig. 5.

Splicing phenotypes of *mss116Δ* strains expressing variants of Mss116p using RT-PCR. (a) Simplified schematic of the *COX1* and *COB* pre-mRNA transcripts. Primers used to amplify the products of aI5 β , aI5 γ , bI1, and bI3 splicing are indicated by arrows. (b) Representative gel images of the products of aI5 β , aI5 γ , bI1, and bI3 splicing after PCR cycle 26. Ligated exons were not amplified in the negative control, in which reverse transcriptase was not included in the cDNA synthesis reaction (-RT). (c) Histograms of the amount of ligated exons in each mutant strain relative to the wild-type strain. The abundance of ligated exons was normalized to the amplification of the *COX3* transcript. Data is the average of two independent experiments, and the error bars represent the range of the values divided by two. Exon ligation

in the strain expressing the vector control alone (E.V.) was very low in abundance or essentially zero and may not be seen on the histogram.

Fig. 6. The R245E Mss116p variant does not rescue the growth or splicing phenotypes of the *mss116Δ* strain. (a) Structural representation of the contacts formed by the Mss116p R245 residue, as predicted by several DEAD-box protein crystal structures (see legend of Fig. 1). Interactions formed by the Q412 residue are shown for reference. (b) CEN6 plasmids containing wild-type (W.T.) and mutant derivatives of Mss116p (K158A, R245E) or the pRS415 vector control (E.V.) were transformed into the *mss116Δ* strains, as described in Figs. 2 and 3. (c) Representative gel images and (d) histograms for intron splicing in each mutant strain, as described in Fig. 5.

Fig. 7.

ATPase activity of wild-type and mutant derivatives of Mss116p. Representative plots of free ADP versus time for reactions with 50 μ M ATP. The curves are linear fits to the data with the slopes equal to the initial velocity (v_o). For kinetic parameters, see Table 1. W.T., wild-type Mss116p; None, no protein added.

Fig. 8.

Unwinding activity of wild-type and mutant derivatives of Mss116p. (a) Representative gel images for unwinding reactions in the presence and absence of ATP at 30 °C. The mobility of the released ^{32}P -DNA oligonucleotide was confirmed by boiling the complex and running that material alongside the starting material (not shown). (b) Representative time course plots for unwinding. Reactions were initiated by the addition of wild-type or mutant protein (1.5 μM final concentration) or protein dilution buffer (None). Aliquots were quenched at time points over a period of 45 min. Data for wild-type Mss116p (W.T.) and the S305A, T307A, and Q412A variants in the presence of ATP was fit to a first-order, double exponential equation: $\text{Fraction Duplex} = A(e^{-kt}) + B(e^{-kt})$ where A and B are the amplitude of duplex in each phase

and k represents the first order rate constants for each phase. Data for W.T. Mss116p in the absence of ATP, R245E in the presence of ATP, and in reactions without protein was fit to a single exponential: Fraction Duplex = $A(e^{-kt})$ where A is the amplitude and k represents the first order rate constant. See Table 2 for values derived from multiple experiments and calculated errors. Unwinding by S305A in the absence of ATP was similar to the rate and amplitude of unwinding by W.T. in the absence of ATP (not shown). Multiple bands in the duplex are due to the addition of non-templated nucleotides to the RNA transcript.

Fig. 9.

The importance of ATP hydrolysis within the context of the proposed models for Mss116p facilitated intron splicing. (a) In this model, Mss116p may act as an ATP-dependent RNA chaperone to unwind short, non-native helices in structured RNAs. Coupled ATP binding and non-specific RNA binding by Mss116p promotes local strand separation. ATP hydrolysis promotes enzyme turnover and the release of a destabilized RNA. The base pairs of the destabilized structure may re-anneal to re-form the non-native structure or completely dissociate and promote RNA folding to a native structure. Inefficient ATP hydrolysis and enzyme turnover could potentially diminish the relative concentration of destabilized RNAs that re-fold to a functional structure, thereby reducing the efficiency of Mss116p-facilitated RNA processing. (b) A second model is based on the suggestion that Mss116p may capture a short lived, single-stranded region of a particular RNA folding intermediate through ATP-dependent RNA binding and promote the ordered folding of the remaining domains of a group II intron RNA.^{13, 16} In theory, this intermediate could follow folding pathways that either lead to a native structure (adjacent, parallel helices) or a non-native structure (anti-parallel helices). ATP hydrolysis and RNA release at the appropriate time within the folding pathway may increase the likelihood that the RNA will form the native, functional structure. If ATP hydrolysis and enzyme turnover are slow, the protein could sterically impede native folding. Consequently, the susceptibility of the RNA to mis-fold into a non-native structure may increase, thus reducing the efficiency of Mss116p-facilitated intron splicing.

Table 1

Kinetic parameters for ATP hydrolysis

Protein	K_m (μM) ^a	k_{cat} (min^{-1}) ^a
W.T.	167 ± 20^b	29.78 ± 3.62^b
K158A	N.D. ^c	0.24^d
R245E	554 ± 153^b	8.18 ± 0.08^{ef}
S305A	162 ± 16^b	19.30 ± 8.49^b
T307A	180 ± 4^b	3.66 ± 0.27^f
Q412A	177 ± 33^b	23.55 ± 5.60^f

^aParameters were measured at 37 °C, as described in Materials and Methods.^bErrors are the standard deviations calculated from 3-7 independent experiments^cN.D., not determined.^dData from one experiment.^eRate measured using 1 mM ATP, but was within error of the rate measured using 0.5 mM ATP^fAverage of two independent experiments with the error estimated by dividing the spread of values by two.

Table 2

Percentage and rates of duplex unwinding

Protein	1 st Phase		2 nd Phase	
	% Unwound	Rate (min ⁻¹)	% Unwound	Rate (min ⁻¹)
None / ATP ^{a,b}	8.4 ± 0.8	0.002 ± 0.0001	N/A ^c	N/A ^c
W.T. ^{a,b, d}	11.5 ± 0.7	0.002 ± 0.0003	N/A ^c	N/A ^c
W.T. / ATP ^{d,e,f}	12.3 ± 0.7	0.22 ± 0.036	34.5 ± 0.4	0.007 ± 0.0004
R245E / ATP ^{b,g}	12.4	0.003	N/A ^c	N/A ^c
S305A / ATP ^{e,f}	12.8 ± 0.4	0.16 ± 0.023	31.1 ± 2.4	0.004 ± 0.0014
T307A / ATP ^{a,f}	1.3 ± 0.2	0.11 ± 0.012	19.0 ± 2.8	0.002 ± 0.0006
Q412A / ATP ^{a,f}	3.4 ± 0.9	0.13 ± 0.002	21.3 ± 1.2	0.003 ± 0.00002

^a Average of two independent experiments with the error estimated by dividing the spread of values by two.

^b Percentage of unwound duplex and rates were obtained from fitting the data to a first order single exponential equation; see Legend to Figure 8.

^c N/A, not applicable

^d W.T., wild-type

^e Errors are the standard deviations calculated from three independent experiments.

^f Percentage of unwound duplex and rates were obtained from fitting the data to a first order double exponential equation; see Legend to Figure 8.

^g Data from one experiment.