The breast cancer tumor suppressor BRCA2-interacting protein, DSS1, and its homologs are critical for DNA recombination in eukaryotic cells. We found that Dss1p, along with Mlo3p and Uap56p, Schizosaccharomyces pombe homologs of two messenger RNA (mRNA) export factors of the NXF–NXT pathway, is required for mRNA export in S. pombe. Previously, we showed that the nuclear pore-associated Rae1p is an essential mRNA export factor in S. pombe. Here, we show that Dss1p and Uap56p function by linking mRNA adapter Mlo3p to Rae1p for targeting mRNA–protein complex (mRNP) to the proteins of the nuclear pore complex (NPC). Dss1p preferentially recruits to genes in vivo and interacts with –FG (phenylalanine glycine) nucleoporins in vivo and in vitro. Thus, Dss1p may function at multiple steps of mRNA export, from mRNP biogenesis to their targeting and translocation through the NPC.

**Introduction**

Studies from various laboratories indicate that messenger RNA (mRNA) is exported by homologs of the heterodimeric NXF–NXT class of non-beta-type carrier proteins (Zenklusen and Stutz, 2001; Cullen, 2003; Stutz and Izaurralde, 2002). Lastly, we showed that Dss1p and Rae1p may function at multiple steps of mRNA export, from mRNP biogenesis to their targeting and translocation through the NPC. Dss1p may function at multiple steps of mRNA export, from mRNP biogenesis to their targeting and translocation through the NPC.

**Keywords**: Dss1p; Mlo3p; mRNA export; Uap56p

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**Received: 31 January 2005; accepted: 19 May 2005; published online: 30 June 2005**
Previously, we described Elf1p as an mRNA export factor in S. pombe (Kozak et al., 2002). Elf1p is an ATPase, and its ability to hydrolyze ATP is essential for its mRNA export function. By using a synthetic lethal genetic screen using an ATPase-deficient elf1-1 mutant S. pombe strain, we identified Dss1p as a novel mRNA export factor. We show that Mlo3p and Uap56p, two key components of NFX–NXT pathway, are also required for normal mRNA export in S. pombe. In addition, we show that Dss1p and Uap56p function by linking mRNA adapter Mlo3p to Rae1p for targeting mRNA–protein complex to the proteins of the NPC. In humans, DSS1 functions with breast cancer tumor suppressor BRCA2 in DNA recombination (Yang et al., 2002). Human DSS1 and S. cerevisiae Sem1p copurify with 19S proteasomes and Sem1p was found associated with double-strand breaks along with proteasomes (Krogan et al., 2004). The identification of Dss1p as an integral component of the mRNA export machinery extends its biological role beyond recombination.

Results

Dss1p is required for mRNA export in S. pombe

A synthetic lethal genetic screen based on elf1-1 mutant strain led to the isolation of several ESL (elf synthetic lethal) strains including ESL49 harboring elf1-1 and a putative esl1-49 mutation. To keep the strain viable, the ESL49 cells expressed the wild-type elf1 gene from the thiamine (vitamin B1)-repressible nmt81 promoter in pREP81X (Maundrell, 1993). ESL49 mutant grew normally in the absence of B1 but did not grow when the expression of elf1 was inhibited by the addition of B1 (Figure 1A). Additionally, poly(A)+ RNA accumulated in the double mutant even when the cells were grown in the absence of B1 (Figure 1Ba and b). Complementation by an S. pombe genomic library identified a plasmid carrying the dss1 gene that was able to rescue growth in +B1 condition (Figure 1A) as well as the mRNA export defects of ESL49 strain under both +B1 (Figure 1Bc and −B1 conditions (not shown). DNA sequences corresponding to dss1 were able to marker rescue the synthetic lethal growth and mRNA export defect phenotypes of ESL49 cells (data not shown). These results demonstrate that dss1 gene carried the esl1-49 mutation and that dss1 was not a multicopy suppressor of ESL49 synthetic lethality.

The dss1 gene (SPAC3G6.02) encodes a 71-amino-acid acidic protein with conserved homologs from yeast to human (Marston et al., 1999). DNA sequence analyses of the dss1 locus in ESL49 cells revealed that a tryptophan residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue at amino acid 26 was changed to a termination residue.
A failure to segregate chromosomes properly in mitosis and caused lethality in *S. pombe* (Javerzat et al., 1996). We found that *mlo3* gene was not essential for growth of *S. pombe* cells although the null strain grew very slowly compared to a wild-type strain (Figure 2A). We next tested the extent of poly(A)⁺ RNA localization in *S. pombe* cells. There was substantial accumulation of poly(A)⁺ RNA in the nucleus of *Dmlo3* cells, suggesting that Mlo3p is important for mRNA export in *S. pombe* (Figure 2Ba). When the *mlo3* mutation was combined with *dss1* or *dss1*-*rae1-167* mutations, the double mutants were viable (Figure 2A). *Dmlo3*-*rae1-167* double mutant cells grew slightly better than *Dmlo3* cells (Figure 2A). In contrast, the *Dmlo3*-*mex67* double mutant was not viable. For growth, *Dmlo3*-*mex67* double mutant cells carried a plasmid (pREP81X-*mlo3*) expressing *mlo3* from a thiamine-repressible *nmt81* promoter. The double mutant cells were able to grow in the absence of B1, but growth was inhibited in the presence of B1 when the expression of *mlo3* was turned off (Figure 2A). We tested the extent of poly(A)⁺ RNA localization in nonlethal double mutant strains *dss1*-*rae1-167* and *Dmlo3*-*mex67*, respectively, with or without B1 on EMM plates. (D) UV sensitivity of *dss1*-*rae1-167* and *Dmlo3* strains. For comparison, wild-type and *rad1-1* (Rowley et al., 1992) strains were used. See Materials and methods for details. (E) mRNA export of strains in (C), as indicated (a–i). (j–r) DAPI-stained DNA.

Figure 1 Growth, UV sensitivity and poly(A)⁺ RNA accumulation in *S. pombe* mRNA export mutants. (A) Synthetic lethal growth and complementation of ESL49 (elf1-1 *dss1-49*/pREP81X-elf1) by *dss1* in the presence (+ B1) or absence (− B1) of thiamine (see Materials and methods for details). (B) Nuclear accumulation of poly(A)⁺ RNA in ESL49 (elf1-1 *dss1-49*/pREP81X-elf1) − B1 and + B1 with vector (a, b). (c) ESL49 complemented with a genomic multicopy plasmid expressing *dss1* in + B1 condition. (d) *dss1-49* complemented by pSEM1. (e–h) Corresponding DAPI-stained cells to visualize DNA. (C) Growth of *dss1* and *dss1-49* and double mutant strains of *dss1* or *dss1-49* with *rae1-167* or *mex67*, respectively, with or without B1 on EMM plates. (D) UV sensitivity of *dss1-49* and *Δdss1* strains. For comparison, wild-type and *rad1-1* (Rowley et al., 1992) strains were used. See Materials and methods for details. (E) mRNA export of strains in (C), as indicated (a–i). (j–r) DAPI-stained DNA.
when the cells were grown under the synthetic lethal conditions (+ B1) (Figure 2B, compare panel f with a). In contrast, there was no additive increase in the amount of poly(A)⁺ RNA in the nucleus of the double mutants Δmlo3 dss1-49, Δmlo3 Δdss1 and Δmlo3 rae1-167 (Figure 2B, compare panels b–d with panel a). These results are consistent with an epistatic relationship among mlo3, dss1 and rae1 gene mutations in mRNA export. Yra1p/ALY functions with NXF–NXT, and in S. cerevisiae, it is essential for mRNA export and cell viability. The nonlethality of Δmlo3 rae1-167 cells indicates that Mex67p may be able to function in the absence of Mlo3p, presumably by directly interacting with RNA and/or by utilizing as yet unidentified adapter protein(s).

**uap56 is required for mRNA export in S. pombe**

We found that uap56 was an essential gene for growth in S. pombe (Figure 2A). For viability, a haploid null strain carried a plasmid (pREP42X-uap56) containing a wild-type copy of uap56 gene under the control of the nmt41 promoter. **In situ** hybridization experiments showed no nuclear accumulation of poly(A)⁺ RNA in Δuap56/pREP42X-uap56 cells grown in the absence of B1 (Figure 2Bg). When uap56 gene expression was turned off by the addition of B1, the cells showed extensive accumulation of poly(A)⁺ RNA in the nucleus (Figure 2Bh). These results show that Uap56p is essential in S. pombe for normal mRNA export.

We wanted to find out the cellular localization of Dss1p, Mlo3p and Uap56p in S. pombe cells. Both Mlo3p-GFP and Uap56p-GFP localized to the nucleus while Dss1p-GFP was found both in the cytoplasm and in the nucleus including a fraction stably associated with the nuclear pore (see Supplementary data for details).

**Dss1p or Uap56p links Mlo3p to Rae1p**

Physical interactions among Mlo3p, Dss1p, Uap56p and Rae1p were tested by using purified proteins made in bacteria. A Mal-Mlo3p fusion protein was unable to bind purified His-Rae1p (Figure 3A, lane 5). However, under the same experimental condition, Mal-Mlo3p was able to bind a GST-Dss1p fusion (Figure 3A, lane 4) but not GST alone (not shown). Moreover, Maltose-binding protein alone did not bind GST-Dss1p (data not shown). Thus, Mal-Mlo3p could directly interact with GST-Dss1p. When GST-Dss1p and His-Rae1p were added to the binding reaction mixture together, Mal-Mlo3p was able to retain both proteins in the bound fraction (Figure 3A, lane 6). These results demonstrate that Rae1p could be present in a ternary complex with Mlo3p dependent on Dss1p. We show later that GST-Dss1p can directly interact with Rae1p and Mlo3p independent of each other. Thus, in the ternary complex, Dss1p most likely acts as a linker between Rae1p and Mlo3p by simultaneously interacting with them.

Next, we wanted to confirm direct interaction between Mlo3p and Uap56p. As expected, purified His-Uap56p was able to directly bind Mal-Mlo3p, but as above, Mal-Mlo3p did not interact with His-Rae1p (Figure 3B, lanes 4 and 5). By comparison with the Mlo3p–Dss1p–Rae1p interaction, the formation of an Mlo3p–Uap56p complex raised the possibility of whether Uap56p could also physically link Mlo3p to Rae1p. When His-Uap56p was added to the binding reaction of Mal-Mlo3p and His-Rae1p, Mal-Mlo3p fusion protein bound His-Rae1p along with His-Uap56p (Figure 3B, lane 6) indicating the formation of a ternary complex (Mal-Mlo3p–His-Uap56p–His-Rae1p). Thus, Uap56p also could connect Mlo3p with Rae1p. These results immediately suggest that Rae1p can directly interact with Uap56p. Taken together, Dss1p and
Uap56p may act as linker proteins between Mlo3p and Rae1p in the formation of specific protein complexes in vitro. In addition, if they act similarly in vivo, then the formation of these complexes could have clear implications about Rae1p function in the nuclear mRNP targeting (see Discussion).

**Uap56p and Dss1p can simultaneously bind Mlo3p or Rae1p but not each other**

The above results led us to investigate the nature of interactions of the linker proteins with Mlo3p or Rae1p. We separately examined the interactions of Rae1p or Mlo3p with Dss1p and Uap56p, respectively. A direct interaction between Dss1p and Rae1p was predicted from the previous binding experiment (Figure 3A, lane 6). Indeed GST-Dss1p fusion protein was able to bind His-Rae1p (Figure 3C, lane 5). Under the same condition, however, GST-Dss1p did not bind His-Uap56p (Figure 3C, lane 6). Thus the linker proteins do not interact with each other directly. When both proteins were added to the binding reaction mixture, we found that GST-Dss1p could pull down His-Uap56p along with His-Rae1p (Figure 3C, lane 7). GST alone was unable to bind any of the proteins in a control binding reaction (lane 8). Taken together, these results demonstrate that Rae1p could simultaneously bind Dss1p and Uap56p.

Next, we set out to test the formation of the complementary complex among Mlo3p, Dss1p and Uap56p. GST-Dss1p

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**Figure 3** *In vitro* protein complex formation by Mlo3p, Dss1p, Uap56p and Rae1p. Presence or absence of a protein in a binding reaction is indicated by (+) or (−) on top of the respective lanes. Individual protein bands are identified with arrowheads. Molecular weight markers are shown. A cartoon below each gel depicts hypothetical ternary complexes. (A) GST-Dss1p links Mal-Mlo3p and His-Rae1p. Inputs of Mal-Mlo3p, GST-Dss1p and His-Rae1p are shown (lanes 1–3). Mal-Mlo3p binds GST-Dss1p (lane 4) but not His-Rae1p (lane 5). Mal-Mlo3p pulls down His-Rae1p in the presence of GST-Dss1p (lane 6). (B) His-Uap56p links Mal-Mlo3p to His-Rae1p. Inputs are shown (lanes 1–3). Mal-Mlo3p binds His-Uap56p (lane 4) but not His-Rae1p as in (A) (lane 5), but pulls down His-Rae1p in the presence of His-Uap56p (lane 6). (C) His-Rae1p binds His-Uap56p and GST-Dss1p simultaneously. Inputs including GST are shown (lanes 1–4). GST-Dss1p binds His-Rae1p (lane 5) but not His-Uap56p (lane 6). GST-Dss1p brings down His-Uap56p in the presence of His-Rae1p (lane 7). GST alone fails to bind either His-Rae1p or His-Uap56p (lane 8). (D) His-Mlo3p binds GST-Dss1p and His-Uap56p simultaneously. Lanes 1–3: Input of individual proteins. GST-Dss1p binds His-Mlo3p directly (lane 4) but not His-Uap56p (lane 5). GST-Dss1p forms a complex with His-Uap56p via His-Mlo3p (lane 6).
was immobilized on agarose beads and mixed with purified His-Mlo3p or His-Uap56p. As shown before, GST-Dss1p did not interact with His-Uap56p but bound His-Mlo3p efficiently (Figure 3D, lanes 4 and 5). In a mixture of three proteins, both His-Mlo3p and His-Uap56p were captured in the bound fraction by GST-Dss1p, demonstrating the formation of a ternary complex (Figure 3D, lane 6). Taken together, these results indicate that the linker proteins can coexist in a complex with Mlo3p or Rae1p. Further, the four proteins may be able to form an interlinked structure in which Dss1p and Uap56p link Mlo3p with Rae1p within a single complex.

**Dss1p binds Mlo3p at Nvr and Cvr sequences**

In order to understand the spatial relationship of Dss1p and Uap56p binding on Mlo3p, we tested which regions of Mlo3p are involved in its interaction with Dss1p. By alignment with the amino-acid sequence of Yra1p, we divided Mlo3p into an N-terminal (1–55 aa) domain, a middle RNA binding domain (RBD; 56–134 aa) and a C-terminal (134–199 aa) region (Figure 4A). Further, the N-terminal region could be subdivided into a conserved N1 (1–15 aa) segment and a variable Nvr (16–55 aa) region known to be required for interacting with Mex67p and RNA (Zenklusen et al, 2001). Similarly, the C-terminal sequences could be subdivided into variable Cvr (134–155 aa) and conserved C2 (156–199 aa) regions.

We found that GST fusions of both the N- and the C-terminal regions of Mlo3p, but not GST alone, could bind His-Dss1p directly (Figure 4B and C, lanes 4 and 5). In contrast, a GST fusion of the Mlo3p RBD was incapable of binding His-Dss1p (Figure 4D, lane 4). In order to further dissect what sequences within the N- and the C-terminal regions are responsible for interacting with Dss1p, we tested GST fusion of the Nvr and the Cvr region of Mlo3p for their ability to bind His-Dss1p. Both fragments were able to bind His-Dss1p (Figure 4E and F, lane 4). When a GST fusion of C2 or N1 was used, they both failed to bind Dss1p (Figure 4G, lane 4, and data not shown). In the context of the full-length protein, whether Nvr and Cvr bind Dss1p independently or synergistically is not known.

**Mex67p can displace Uap56p from Mlo3p**

We found that, like S. cerevisiae Yra1p, a GST fusion of Mlo3p-C2 (156–199 aa) could directly bind His-Mex67p while GST alone could not (Figure 4H, compare lane 4 with 5). Since C2 does not contain the Cvr region, this result shows that Mex67p could bind Mlo3p outside the variable region. Similarly, a GST fusion of Mlo3p-N could also interact with His-Mex67p (data not shown). Further, GST fusion of both C2 and N could separately bind His-Uap56p (data not shown). These results confirm that S. pombe Mlo3p behaves similar to Yra1p or ALY in its ability to interact with Mex67p and Uap56p proteins.

It is known from previous studies that Mex67p could displace sub2p from Yra1p (Strasser and Hurt, 2001). It was suggested that binding of Mex67p or Sub2p on Yra1p is exclusive in nature. To test whether the S. pombe proteins exhibit similar properties, a competition experiment was set up. First, the efficiency of His-Mex67p–GST-Mlo3p interaction was tested. When an equimolar quantity of His-Mex67 was mixed to bead-bound GST-Mlo3p, His-Mex67p was retained inefficiently but reproducibly (Figure 4I, lane 1). For testing the competitive binding of His-Uap56p and His-Mex67p to GST-Mlo3p, a large molar excess of His-Uap56p was added to bead-bound GST-Mlo3p to saturate the binding by Uap56p molecules. After incubating the binding reaction for 1 h, the reaction was split into three equal parts. The first part was untreated. To the second part, equimolar His-Mex67p was added. To the third part, five-fold excess of His-Mex67p was added. After further incubation, the binding reactions were washed and bead-bound fractions were loaded onto SDS–polyacrylamide gel in loading dye. When excess His-Uap56p was added, over-stoichiometric quantity of the protein was bound to GST-Mlo3p under the conditions of this experiment. In the challenge experiment, when low (1 ×) quantity of His-Mex67p was added, GST-Mlo3p interacted with both His-Uap56p and His-Mex67p, even though His-Mex67p bound rather inefficiently. The binding reaction may represent a mixture of binary complexes such as GST-Mlo3p–His-Mex67p and GST-Mlo3p–His-Uap56p. Alternatively, the binding may represent a ternary complex of His-Uap56p–GST-Mlo3p–His-Mex67p since His-Uap56p and His-Mex67p did not interact with each other directly (data not shown). When excess His-Mex67p was added, the entire amount of His-Uap56p bound to GST-Mlo3p was lost with a concomitant increase in the amount of His-Mex67p. These results concur with previous findings with S. cerevisiae or metazoan proteins and demonstrate that S. pombe Mex67p and Uap56p bind Mlo3p via the same region and that Mex67p could displace Uap56p from Mlo3p. However, at comparable protein concentration, these results are consistent with the same molecule of Mlo3p simultaneously binding both Uap56p and Mex67p presumably via the two ends. A similar result was obtained previously in the binding studies of Yra1p, Sub2p and Mex67p (Figure 5C, lane 2 of Strasser and Hurt, 2001).

**Mex67p and Dss1p binding on Mlo3p is noncompetitive**

In an analogous competitive experiment, we tested whether binding of Dss1p to Mlo3p could withstand a challenge from His-Mex67p or His-Uap56p. For this purpose, we chose GST fusion of Mlo3p-C to which His-Dss1p and His-Mex67p bind via Cvr and C2 regions, respectively. As expected, GST-Mlo3p-C bound His-Dss1p and His-Mex67p (Figure 4J, lanes 1 and 2). When an equimolar amount of His-Mex67 was added to bead-bound GST-Mlo3p–His-Dss1p, both proteins were retained (Figure 4J, lane 3). But importantly, the amount of His-Dss1p bound to GST-Mlo3p–C remained unchanged upon challenge from His-Mex67p. Similarly, a five-fold excess His-Mex67p did not reduce the quantity of His-Dss1p bound to GST-Mlo3p–C (Figure 4J, lane 4). Increased His-Mex67p did not significantly increase its own binding either, presumably reflecting inefficient binding by the protein. Taken together, these results suggest that the binding of Dss1p and Mex67p (or Uap56p) occurs independently on GST-Mlo3p–C and that the binding of His-Mex67p may not significantly alter the structure of GST-Mlo3p–C to affect its interaction with His-Dss1p. Finally, we also tested for a possible direct interaction between GST-Dss1p and His-Mex67p. GST-Dss1p was unable to bind His-Mex67p (Figure 4K, lane 3). Western analysis with anti-Mex67p anti-
body confirmed the lack of direct binding between the two proteins (Figure 4K, lower panel).

**Rae1p, Uap56p, Mlo3p and Nup159p but not Mex67p associate with Tap-tagged Dss1p in *S. pombe* extract**

We wanted to know whether the observed interactions in vitro represent bona fide functional interactions in vivo. An *S. pombe* strain was constructed in which the genomic *dss1* gene was replaced by a *dss1-tap* gene fusion. As a negative control for the experiment, Tap was expressed in wild-type *S. pombe* cells from a plasmid (pNTAP41) carrying the *nmt41* promoter. Extracts containing Dss1p-Tap or Tap alone were incubated with IgG-Sepharose beads, and following cleavage of the tag with TEV-protease, bound protein fractions were analyzed by Western blot analyses. Dss1p-Tap was able to interact with cellular proteins that were recognized by polyclonal antibodies against Mlo3p, Uap56p, Rae1p and Nup159p (Figure 5A, lane 4). In contrast, in the control experiments, Tap alone did not associate with these proteins. Interestingly, Mex67p and Nsp1p (data not shown) did not interact with Dss1p-Tap. Nup98p was also tested but the protein degraded very rapidly during the preparation of the

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**Figure 4** GST-Mlo3p interaction with His-Dss1p, His-Mex67p and His-Uap56p. (A) Schematic diagram of amino-acid residue positions on Mlo3p indicating various regions by comparison with Yra1p primary amino-acid sequence (Zenklusen et al., 2001). Approximate locations of Dss1p and Uap56p binding sites on Mlo3p are shown on top of Mlo3p sequence. GST fusions of Mlo3p are indicated. N: N-terminal sequence (1–55 aa); NVr: variable sequence (16–54 aa); N1: conserved sequence (1–15 aa); RBD: RNA binding domain (56–134 aa); C: C-terminal region (135–199 aa); CVr: variable sequence (135–157 aa); C2: conserved region (158–199 aa). (B–G) Binding of His-Dss1p by GST fusions of Mlo3p as indicated in each gel. Lanes 1–3: Input. Lanes 4 and 5: Binding and negative control. Individual bands are identified with arrowheads and molecular weight markers are shown on the left-hand side. (H) Binding of His-Mex67p by GST-Mlo3p-(C2). Lanes 1–3: Input. Lane 4: GST-Mlo3p-(C2) binds His-Mex67p. Lane 5: GST control. (I) Displacement of prebound His-Uap56p from GST-Mlo3p by His-Mex67p. Lane 1: Binding of GST-Mlo3p to His-Uap56p. Lane 2: Binding of His-Uap56p to GST-Mlo3p. The effect of the addition of 1 × (lane 3) or 5 × (lane 4) molar amounts of His-Mex67p to prebound fractions of GST-Mlo3p and His-Uap56 (lane 2) is shown. (J) Noncompetitive binding of His-Mex67p and His-Dss1p on GST-Mlo3p-(C). GST-Mlo3p-(C) binds His-Dss1p (lane 1) or His-Mex67p (lane 2) or both (lane 3). Addition of five-fold excess Mex67p for the dissociation of His-Dss1p from GST-Mlo3p (lane 4) is shown. (K) GST-Dss1p does not bind His-Mex67p. Extract containing His-Mex67p was added to GST-Dss1p (lane 3) or GST alone (lane 4). Bottom panel: Western blot of a duplicate gel probed with anti-Mex67p antibody. In (J), * indicates a copurifying contaminant.
These results are consistent with the observed direct interaction between Dss1p and Mlo3p or between Dss1p and Rae1p and an indirect interaction of Dss1p with Uap56p via Mlo3p or Rae1p. The absence of Mex67p in the pull-down is also in agreement with its lack of direct binding to Dss1p in vitro. However, in contrast to its interaction with Mlo3p–Dss1p complex in vitro, Mex67p may not be able to associate with functional, Mlo3p-containing complexes of Dss1p in vivo. The ability of Dss1p–Tap to associate with Nup159p indicates that Dss1p may interact with the NPC proteins directly or indirectly. Recently, Tap-tagged Sem1p and human DSS1 were shown to copurify a number of proteasomal subunits (Krogan et al., 2004). But interestingly, no mRNA export factor was found associated with either protein. It is possible that under the purification conditions, only a minor fraction of Sem1p/DSS1 is associated with mRNA export factors. Alternatively, DSS1/Sem1p may be part of a minor mRNP complex in these organisms.

**Figure 6** mRNP targeting in *S. pombe* and *S. cerevisiae*. In *S. pombe*, the progression of mRNP formation starts following recruitment of Uap56p, Mlo3p and Dss1p. Direct interaction of Uap56p and Dss1p with Rae1p connects Mlo3p to the NPC (mRNA cargo not shown). Presumptive interactions between Dss1p inside the complex and –FG sequences of Nup98p are shown based on their direct interaction in vitro. At the nuclear pore, Rae1p associates with Nup98p (our unpublished results). Additional proteins may be involved in the process in vivo (not shown). For comparison, the major mRNA export pathway in *S. cerevisiae* via NXX–NXT homolog Mex67p–Mtr2p is shown. Unlike in *S. cerevisiae*, NXX–NXT pathway plays a minor role in *S. pombe* (see text for details).

**Dss1p binds –FG-containing regions of Nup159p and Nup98p but not Nsp1p**

*In vivo* pull-down results led us to test whether Dss1p could interact directly with Nup159p. We used a –FG (phenylalanine glycine)-containing region of *S. pombe* Nup159p (481–850 aa) that directly binds Mex67p sequences (Thakurta et al., 2004). His-Nup159p could successfully bind GST-Ds1p while it could not bind GST alone (Figure 5B, lanes 4 and 5). We then tested –FG-containing region of Nup98p (141–612 aa) for its ability to interact with Dss1p. GST-Dss1p could bind the Nup98p fragment (lane 9) but GST alone did not bind the protein (lane 10). However, under the same experimental conditions, GST-Dss1p could not bind Nsp1p (lane 14), a ubiquitous –FG-containing protein (homolog of vertebrate p62). We also found that GST-Dss1p did not bind *S. pombe* Npp106p (data not shown). Thus, in addition to Mlo3p and Rae1p, Dss1p appears to be able to directly interact with the –FG-containing regions of two NPC components whose functions are critical in mRNA export.
Dss1p preferentially recruits to genes

In vivo association of Dss1p with Mlo3p prompted us to ask whether the protein recruited early to transcriptionally active genes. Using an HA-tagged dss1 strain, we analyzed by chromatin immunoprecipitation (ChiP) the amount of DNA from a transcribed region and an intergenic region that associated with Dss1p. There was a significant amplification of DNA from the transcribed region of nmt1 gene as compared to the mock immunoprecipitation experiment (Figure 5C, upper panel, compare lane 3 with 2). In contrast, similar amount of DNA amplified from an intergenic region by the HA antibody or by the mock immunoprecipitation (Figure 5C, lower panel, compare lane 3 with 2). These results suggest that Dss1p recruited preferentially to genes, and presumably to transcripts, over untranscribed intergenic regions of the chromosome.

Discussion

In this study, we identified Dss1p as a novel mRNA export factor in S. pombe. We show that it associates with genes and together with Uap56p links mRNA adapter Mlo3p to Rae1p for targeting the mRNP complex to the NPC and likely participates in the translocation of the cargo through the NPC. Homologs of Uap56p and Mlo3p are known components of mRNA export in other organisms, but this is the first demonstration of their direct role in mRNA export in S. pombe. Finally, we describe how Rae1p could function in mRNA export by associating with proteins of the mRNP complex.

An mRNP-targeting complex in S. pombe

Previous studies indicated a direct role of Rae1p homologs in the mRNA export process but they did not demonstrate a direct interaction of Rae1p homologs with the soluble mRNP machinery. Our results are consistent with the proposal that Rae1p facilitates the docking of mRNP to the nuclear pore via Nup98p. Further, in vitro binding results imply that by combining two ternary interactions (Rae1p–Uap56p–Mlo3p and Rae1p–Dss1p–Mlo3p), a single complex may form in vivo for targeting mRNPs to the NPC.

How is mRNP targeted in S. pombe? For a comparative analysis, current understanding of the NXF–NXT-mediated mRNP targeting in S. cerevisiae/human and the Rae1p-mediated process in S. pombe is presented in Figure 6. In the NXF–NXT pathway, Sub2p/Uap56p recruits to elongating transcripts by interacting with the THO complex (Zenklausen et al., 2002). Sub2p/Uap56p then recruits Yra1p/Aly by directly interacting with the protein. Subsequently, NXF–NXT heterodimer displaces Sub2p/Uap56p and directly binds Yra1p/Aly and links the mRNP by interacting with −FG sequences of Nup98p at the NPC. The initial events may be similar in S. pombe, although the recruitment of Uap56p may be mechanistically different, as components of the THO complex appear to be absent in the fission yeast. Next, Uap56p could recruit Mlo3p–Dss1p together onto the transcripts. Alternatively, Dss1p could be recruited only after Mlo3p is recruited. In contrast to being displaced by NXF–NXT, Dss1p and Uap56p remain associated with Mlo3p and directly link it to Rae1p on the nuclear pore. Rae1p is likely bound to Nup98p via the latter’s Rae1p binding (GLEBS) domain.

In S. pombe, the functions of Mex67p–Nxt1p are critical in the presence of a mutant Rae1p. How does Mex67p–Nxt1p function in S. pombe cells? One possibility is that in the mutant Rae1p background, Uap56p is displaced by Mex67p–Nxt1p to target the mRNP. Another possibility is that Mex67p–Nxt1p associates with the Uap56p–Mlo3p–Dss1p complex without displacing Uap56p. If so, it will be expected to associate with the mRNP at the NPC.

Role of Uap56p and Dss1p in mRNP targeting

Both Rae1p and NXF–NXT appear to function with Mlo3p/ Yra1p/ALY. However, there are clear differences in their mechanisms and requirements for mRNP targeting to the NPC. In the Rae1p complex, Dss1p and Uap56p simultaneously link Mlo3p (hence the mRNP) to Rae1p in the nucleoplasm or at the NPC. Thus, Uap56p and Dss1p remain associated with Mlo3p when the mRNPs are connected to Rae1p. This requires Uap56p to play a critical role beyond the initial recruitment of Mlo3p. In S. cerevisiae, Sub2p affects recruitment of Yra1p on intron-containing genes but not on intron-less genes (Lei and Silver, 2002b). However, Sub2p is required for the export of both kinds of mRNA (Strasser and Hurt, 2001). Thus, it must have a role in addition to Yra1p recruitment in mRNA export. A direct interaction of Sub2p with Gle2p may provide such a role for linking some mRNAs to Gle2p for export.

Dss1p binds Mlo3p directly and the latter binds both Mex67p and Uap56p. We expected that Mex67p would associate with Dss1p complex in vivo. However, Uap56p, but not Mex67p, was found associated with Dss1p complex in the S. pombe extract. Since Mlo3p binds Uap56p and Mex67p in an exclusive manner (Figure 4I), one possible reason for this result could be a much higher cellular concentration of Uap56p as compared to Mex67p in S. pombe cells. Notwithstanding the cause, the presence of Uap56p within the Dss1p complex clearly suggests that it remains associated with Mlo3p when the mRNP complex interacts with Rae1p. In further support of this possibility, in C. tentans, Uap56p homolog HEL was shown to remain associated with mRNPs until they reach the NPC (Kiesler et al., 2002).

The multicopy expression of dss1 did not rescue the mRNA export defect associated with ∆uap56/pREP42X-uap56 strain; conversely, the multicopy expression of uap56 could not rescue the mRNA export defect of ∆dss1 strain (data not shown). Even though each protein could form a link between Mlo3p and Rae1p in vitro, the partial complexes could not adequately function in mRNA export in vivo. Thus, linking Mlo3p and Rae1p is not sufficient for exporting mRNA. One possibility is that both Dss1p and Uap56p are necessary for the formation of the proper targeting complex. Another, not mutually exclusive, possibility is that targeting of some mRNPs specifically requires Dss1p or Uap56p.

Significance of Dss1p–FG interaction

Dss1p may participate at many steps in the mRNA export process. Its recruitment to genes indicates a possible early role. A second likely role is in the formation of the targeting complex. A third functional role of Dss1p was suggested from its direct interaction with the −FG-containing fragments of Nup159p and Nup98p. In this regard, it could function like Mex67p–Nxt1p. However, multicopy expression of dss1 did not rescue the mRNA export defect either of rae1-167 at
Table I  S. pombe strains used in this study

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<th>Strain</th>
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<td>Yoon et al (2000)</td>
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<tr>
<td>Wild type</td>
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<td>Yoon et al (2000)</td>
</tr>
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<td>Kozak et al (2002)</td>
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<td>Yoon et al (2000)</td>
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Materials and methods

Strains and culture

Basic genetic and cell culture techniques used have been described previously (Moreno et al, 1991; Alfa et al, 1993). Strains are described in Table I. The null strains for Uap56, Adss1 and Δmlo3 were constructed by standard gene disruption techniques. Uap56 null strain was constructed in a diploid strain in the presence of a plasmid and was confirmed by Southern blot analyses.

Spot assay for growth, UV sensitivity and in situ hybridization

Growth and in situ hybridization methods were described before (Amberg et al, 1992; Yoon et al, 2000; Thakurta et al, 2004). For UV sensitivity, growing cultures (OD0.5) were serially diluted and plated on YEA medium and exposed to various doses of UV (75–150 J/m2). Growth was monitored after 3 days at 30°C.

Construction of plasmids

ds1 genomic clone was isolated from a genomic library. cDNA clone of ds1 was made by RT–PCR from total RNA made from wild-type S. pombe cells. The gene for Uap56 was amplified from the genome and inserted into pREP42X vector (Maundrell, 1993). C-terminal GFP fusions of S. pombe genes were constructed in a Ura+ promoter-less vector into which respective genes including its promoter were cloned. The promoter was placed with their 5' end fused to DNA sequences expressing GFP. The construction of plasmids for bacterial expression of the N-terminal GST fusion proteins was made in pGEX4T-3 or pGEX4T-3 (Amersham). His-tagged proteins were made by inserting cDNA or genomic DNA of yeast into pET11b vector (Novagen, CA). For Mal fusion of Mlo3p, its coding cDNA was inserted into pMal2X (New England Biolabs, Beverly, MA). For various GST-Mlo3p deletions, appropriate cDNA fragments representing various domains were expressed similar to the full-length protein. Other plasmids were described before (Yoon et al, 2000; Kozak et al, 2002).

Purification and binding of bacterial proteins

For His-tagged protein purification, a Talon column was used; for GST fusion, a GSH-Sepharose bead was used; and Mal-Mlo3 was purified on amylose resin. Proteins were eluted in 500 mM imidazole (His proteins), 20 mM glutathione (GST fusions) and 10 mM Maltose (Mal-Mlo3 fusion). Eluted proteins were dialyzed into the universal binding buffer (Kunzler and Hurt, 1998) and stored at –70°C in aliquots. Binding reactions were performed in the universal binding buffer in Talon matrix (Clontech), GSH-Sepharose beads or amylose resins with 5–10 μg of purified proteins. Bound proteins were separated on 4–12% NuPAGE gels, and stained by Simply Blue (Invitrogen). For Western blot analyses, 10% of the reaction was run on 4–12% NuPAGE (Invitrogen, CA) gels and transferred to a PVDF membrane.

Antibodies and Western blot detection method

Polyclonal antibodies to S. pombe Mlo3p and Uap56p were made by expressing full-length proteins in bacteria and injecting them into rabbits. Nup159 (450–830 aa) of S. pombe was expressed from pET11b vector for raising antibody. Rae1p and Mex67p antibodies were described before (Bharathi et al, 1997; Yoon et al, 2000). GST fusion proteins were detected by using a monoclonal anti-GST antibody (BABC0). Standard chemiluminescent detection methods (NEW, MA) were used to detect proteins.

Immunoprecipitation

An S. pombe strain expressing Dss1-TAP fusion from its chromosomal locus was constructed based on published method (Gould et al, 2004). For control experiments, cells transformed with pNTAP41 were used. Purification of Tagged proteins from S. pombe cells was based on published methods except for the following modification. Extracts were made in buffer containing 0.5% Triton and 250 mM KI and proteins bound to IgG-Sepharose beads. The complexes were cleaved from the beads using TEV-protease and the bound proteins analyzed by gel electrophoresis.

We performed ChIP by following published methods (Takahashi et al, 2000) using a monoclonal antibody to HA epitope. Gene-specific PCR primers were used to amplify the 3' end of nmt1 gene. A pair of primers corresponding to untranslated portion of S. pombe chromosome III was used for the intergenic region. Sequences of primers are available on request.

Supplementary data

Supplementary data are available at The EMBO Journal Online.

Acknowledgements

We thank Dr A Ashworth and Dr K Gould for providing us with strains and plasmids, and Drs Alain Mir, J Landry and D Chatteraj for critically reading the manuscript.
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Lei EP, Silver PA (2002b) Intron status and 3'-end formation control cotranscriptional export of mRNA. *Genes Dev* 16: 2761–2766


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