

## *Schizosaccharomyces pombe* *rsm1* Genetically Interacts with *spmex67*, Which Is Involved in mRNA Export

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**We have previously isolated three synthetic lethal mutants from *Schizosaccharomyces pombe* in order to identify mutations in the genes that are functionally linked to *spmex67* with respect to mRNA export. A novel *rsm1* gene was isolated by complementation of the growth defect in one of the synthetic lethal mutants, SLMex1. The *rsm1* gene contains no introns and encodes a 296 amino-acid-long protein with the RING finger domain, a C3HC4 in the N-terminal half. The  $\Delta rsm1$  null mutant is viable, but it showed a slight poly(A)<sup>+</sup> RNA accumulation in the nucleus. Also, the combination of  $\Delta rsm1$  and  $\Delta spmex67$  mutations confers synthetic lethality that is accompanied by the severe poly(A)<sup>+</sup> RNA export defect. These results suggest that *rsm1* is involved in mRNA export from the nucleus.**

**Key words:** *rsm1*, *mex67*, synthetic lethality, mRNA export, *Schizosaccharomyces pombe*

In eukaryotes, nucleocytoplasmic transport of macromolecules generally requires soluble transport receptors that specifically recognize and transport their cargoes through nuclear pore complexes (NPCs) embedded in the nuclear envelope (Mattaj and Englmeier, 1998; Strasser and Hurt, 1999). Translocation of proteins and nuclear export of rRNAs, tRNAs, snRNAs through the NPCs are mediated by transport receptors that belong to the importin- $\beta$  type receptor family. These receptors mediate transport by transiently interacting with the phenylalanine-glycine (FG) repeats of a class of nucleoporins that line the channel of the NPC. The directionality of transport is controlled by the small GTPase Ran (Lei and Silver, 2002; Weis, 2002; 2003).

As the export of mRNA appears not to be directly dependent on RanGTP and the receptors of importin- $\beta$  family, this process is significantly complicated and is different from the other nucleocytoplasmic transport routes mentioned above (Reed and Hurt, 2002). Beginning with transcription, mRNAs are bound and packaged by various RNA binding proteins to form messenger ribonucleoprotein (mRNP) complexes. During transcription, the nascent pre-mRNA is capped at the 5' end, introns are removed by splicing, and the 3' end is cleaved and polyadenylated. Only the export-competent mRNP complexes with mature mRNAs are then exported through the NPCs. It has become apparent that mRNA export is tightly coupled to several steps of gene expression (Zenklusen and Stutz, 2001; Dreyfuss *et al.*, 2002; Maniatis and Reed, 2002;

Reed and Hurt, 2002; Weis, 2002).

One of the most important mRNA export factors is the heterodimer of the large subunit, Mex67, and the small subunit, Mtr2, in yeast, whose conserved metazoan counterparts are known as TAP/NXF1 and p15/NXT, respectively. The larger subunits of these heterodimers, Mex67/TAP, belong to members of the evolutionally conserved family of NXF proteins (Conti and Izaurralde, 2001). These mRNA export receptors in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* are essential for viability and the export of mRNA (Segref *et al.*, 1997; Tan *et al.*, 2000; Herold *et al.*, 2001). Mex67/TAP binds to mRNA, shuttles between the cytoplasm and the nucleus, and interacts with FG repeat-containing nucleoporins, although it does not belong to the importin- $\beta$  receptor family and does not appear to require a Ran system for binding and releasing of its cargo, mRNA (Conti and Izaurralde, 2001; Reed and Magni, 2001). Structural studies of Mex67/TAP exhibit a pronounced modular organization (Conti and Izaurralde, 2001). The N-terminal half recognizes mRNA export cargoes and consists of a RNA binding domain (RBD) and a leucine-rich repeat (LRR) domain (Liker *et al.*, 2000). The C-terminal half binds to the NPC and comprises of a NTF2-like domain and an UBA-like domain (Fribourg *et al.*, 2001; Grant *et al.*, 2003).

In *Schizosaccharomyces pombe*, *mex67* (*spmex67*) is not essential, and its involvement in mRNA export is revealed only by its synthetic lethality with a mutation of *rae1* that is essential for growth and mRNA export (Brown *et al.*, 1995; Yoon *et al.*, 2000). However, spMex67p also binds to mRNA, shuttles between the

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nucleus and the cytoplasm, and functions to export mRNA. Moreover, *spmex67* expressed from a multicopy plasmid can partially suppress the mRNA export defect of *rae1-167* mutation, and the overexpression of spMex67p in wild type cells inhibits mRNA export (Yoon *et al.*, 2000). In order to identify mutations in genes that are functionally linked to *mex67* in mRNA export, three synthetic lethal mutants with the *spmex67* null allele present in *S. pombe* were isolated, and under synthetic lethal conditions, these mutants exhibited an accumulation of poly(A)<sup>+</sup> RNA in the nucleus (Yoon, 2003).

In this study, we described the isolation of the novel *rsm1* gene by complementation of the growth defect in one of the synthetic lethal mutants. In addition, the *rsm1* deletion mutants were shown to cause defects in the process of mRNA export, especially when combined with *spmex67* null allele. These results suggested that *rsm1* is involved in the mRNA export in *S. pombe*.

## Materials and Methods

### Strains and culture

The basic genetic and cell culture techniques used have been described (Moreno, 1991; Alfa, 1993). The *S. pombe* strains used were of wild type AY217: *h<sup>-</sup> leu1-32 ura4-d18*; SP286: *h<sup>+</sup>/h<sup>+</sup> leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-210/ade6-216* (Beach and Matsumoto, 1991); SLMex1: *h<sup>-</sup> leu1-32 ura4-d18 Δspmex67::kan/pREP81X-spmex67<sup>+</sup>* (Yoon, 2003); Δspmex67: *h<sup>-</sup> leu1-32 ura4-d18 Δspmex67::kan/pREP81X-spmex67<sup>+</sup>* (Yoon *et al.*, 2000); and Δrsm1: *h<sup>-</sup> leu1-32 ura4-d18 Δrsm1::ura4* (this work). Appropriately supplemented EMM medium was used to express genes from the pREP plasmids containing the *nmt* promoter (Maundrell, 1993). The *nmt* promoter was repressed by the addition of 10 μM thiamine in EMM medium (Forsburg, 1993).

### Isolation of *rsm1*

SLMex1 (*h<sup>-</sup> leu1-32 ura4-d18 Δspmex67::kan/pREP81X-spmex67<sup>+</sup>*) cells were transformed with a partial *Sau3A* genomic library that was cloned into the *SalI* site of pUR18 (Barbet, 1992). Ten transformants were isolated, which could grow on plates in the presence of thiamine at 28°C. The plasmids were rescued from these transformants, amplified in *Escherichia coli*. Four plasmids were eliminated from further investigation because they carried the *spmex67* gene. The remaining six plasmids were re-transformed into SLMex1 for confirmation. Finally, three plasmids that could complement SLMex1 were obtained. The DNA sequence obtained from these plasmids was used to search the *S. pombe* genome database ([http://www.sanger.ac.uk/Projects/S\\_pombe/](http://www.sanger.ac.uk/Projects/S_pombe/), Sanger Center, UK).

### Construction of *rsm1* null

The Δ*rsm1::ura4* null mutation was constructed by first

cloning the *rsm1* gene into a pBluescript SK(+) vector (Stratagene, USA). A deletion between the amino acids 9 and 293 with a *NotI* site placed at the deletion junction was constructed by PCR mutagenesis. A *NotI* fragment bearing *ura4* was inserted into the deletion junction. This plasmid was cut with *BamHI* and *PstI*, and the Δ*rsm1::ura4* fragment was gel-purified and was transformed into the SP286 diploid strain. Ura<sup>+</sup> transformants were selected and screened by Southern blotting for the disruption of one of the *rsm1* loci. The strain was sporulated, and ten tetrads were dissected. All spores formed colonies and showed a 2:2 segregation of *ura4<sup>+</sup>*.

### In situ hybridization

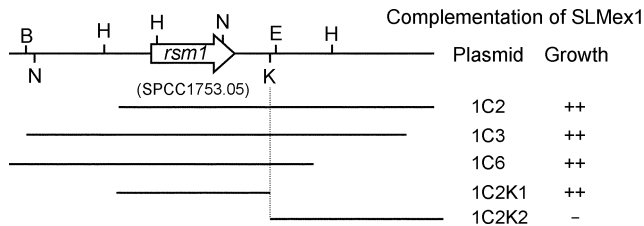
*In situ* hybridization was performed as previously described (Yoon, 2003). Oligo-(dT)<sub>50</sub> carrying an α-digoxigenin at the 3' end was used as the hybridization probe. FITC-anti-digoxigenin Fab antibody (Roche Applied Science, Germany) was used for detecting the hybridization probe by fluorescence microscopy. 4',6-Diamidino-2'-phenylindole (DAPI) was used for observing DNA.

## Results and Discussion

### Cloning of the cognate synthetic lethal gene in SLMex1

We previously reported the isolation of three synthetic lethal mutants, SLMex1, SLMex2, and SLMex3, and mutations that are synthetically lethal with the *spmex67* deletion allele in these mutants defined separate complementation groups (Yoon, 2003). These synthetic lethal mutants are kept viable by the expression of the *spmex67<sup>+</sup>* gene from the plasmid, pREP81X-*spMex67*, under the control of a weak thiamine-repressible *nmt1* promoter (Basi *et al.*, 1993). In the absence of thiamine (-B1), the *spmex67<sup>+</sup>* gene is expressed and the synthetic lethal mutant cells grow normally. The repression of spMex67p expression in synthetic lethal mutant cells by the addition of thiamine (+B1) results in a growth defect that is accompanied by poly(A)<sup>+</sup> RNA accumulation in the nucleus (Yoon, 2003). We have used SLMex1 in this study.

The cognate gene carrying the synthetic lethal mutation in SLMex1 was isolated from a partial *Sau3A* library through functional complementation, as mentioned in Materials and Methods. Three genomic clones, 1C2, 1C3, and 1C6 that allowed growth of SLMex1 when *spmex67<sup>+</sup>* was repressed by the addition of thiamine, were isolated (Fig. 1). Restriction enzyme digestion of the three genomic clones revealed that they had common restriction enzyme fragments. These genomic clones were sequenced at both ends, and the DNA sequence obtained was used to search the *S. pombe* genome database. An identical sequence of each clone was found in the cosmid SPCC1753 (chromosome III), and an overlapping region



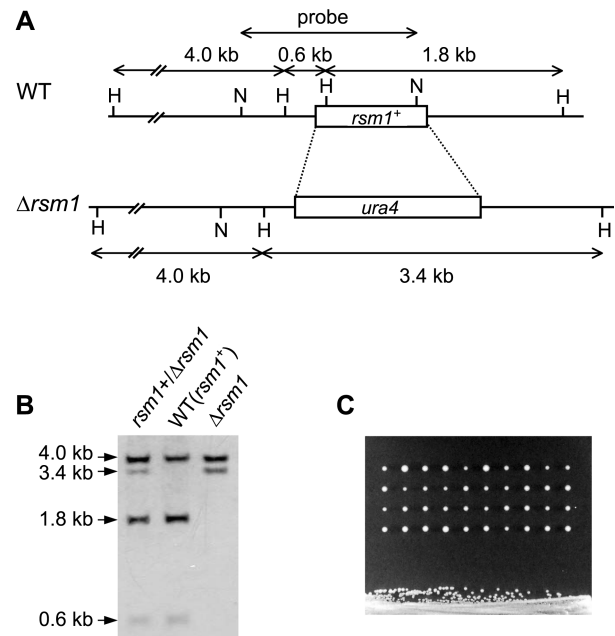
**Fig. 1.** Complementation of SLMex1 and a restriction map of the region surrounding the *rsm1* gene. An open arrow indicates the extent and direction of the *rsm1* open reading frame. The original clone and subcloned fragments, which were tested for the complementation of SLMex1, are shown as horizontal bars. SLMex1 cells carrying pREP81X-mex67 were transformed with different plasmids and were spread on EMM agar in the presence of thiamine. Growth was monitored for 5 days. ++, normal growth; -, no growth. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nco*I.

of three clones contained only one complete ORF, SPCC1753.05 (Fig. 1). To confirm that this ORF is capable of complementing SLMex1, two subclones of 1C2 were constructed. As shown in Fig. 1, 1C2K1, which carried a region from the *Kpn*I site to the end of the 1C2 clone containing the SPCC1753.05 ORF, was able to functionally complement the growth defect of SLMex1. Whereas, 1C2K2, which carried a remaining region from the *Kpn*I site to the other end of 1C2 without this ORF, could not restore the growth defect of SLMex1.

The SPCC1753.05 gene contains no intron and encodes a 296 amino-acid-long protein with predicted molecular weight of 34.6 kDa. We will refer to this *S. pombe* gene as *rsm1*, because of the mRNA export defect and synthetic lethality with *spmex67*. *S. pombe* GeneDB revealed that the N-terminal region of Rsm1p (amino acids 71 to 117) contains the RING finger domain, C3HC4. It has become clear that most, but probably not all, proteins that contain a RING finger domain function as ubiquitin-protein ligases (E3) in many organisms (Fang *et al.*, 2003). However, the region of Rsm1p that is classified as RING finger domain does not contain all conserved cysteine or histidine residues. A BLASTP search of the protein databases revealed that the hypothetical Rsm1p protein showed no significant sequence similarity to known proteins of other organisms.

#### The *rsm1* gene is not essential for growth

In order to determine the phenotype of the *rsm1* knockout, a null mutant in a  $h^+/h^+$  diploid strain was constructed by replacing the *rsm1*-coding region with an *ura4* gene using a one-step gene disruption method (Fig. 2A). The stable *Ura*<sup>+</sup> transformants were screened by Southern blotting for the replacement of one of the *rsm1* locus in diploid cells (Fig. 2B). The diploids were allowed to sporulate, and ten tetrads were dissected. All spores formed colonies, and the *ura4* marker segregated at a ratio of 2:2 (Fig. 2C). Haploid cells carrying the  $\Delta rsm1::ura4$  allele were



**Fig. 2.**  $\Delta rsm1$  deletion mutants are viable. (A) A schematic diagram representing the constructs of the *rsm1* null allele in *S. pombe*. Most of the *rsm1* open reading frame region was substituted with the marker gene, *ura4*. (B) Confirmation of the disruption of the *rsm1* locus. Genomic DNAs isolated from wild type (WT), Diploid disrupted one of the *rsm1* locus ( $rsm1^+/\Delta rsm1$ ), and  $\Delta rsm1$  haploid strains were digested with *Hind*III. A 1.85 kbp *Nco*I fragment used as probe is denoted in (A). (C) Tetrad analysis. Diploid cells disrupted one of the *rsm1* locus were sporulated, and 10 tetrads were dissected on YES plates and were incubated for 3 days at 28°C.

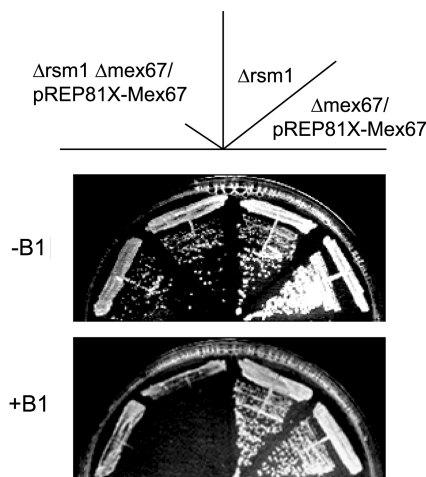
confirmed again by Southern blotting (Fig. 2B). This result indicated that the  $\Delta rsm1::ura4$  allele is not lethal to cells.

#### The *rsm1* is genetically linked with *spmex67*

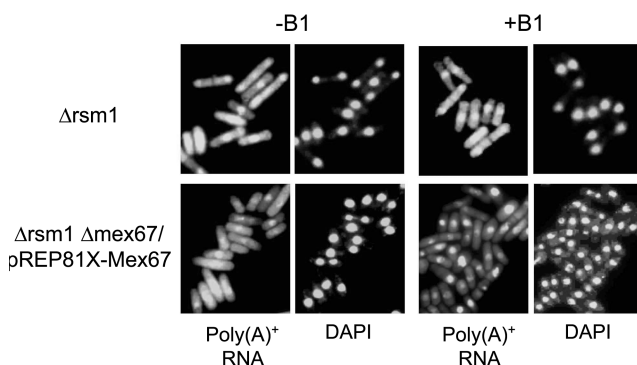
To test whether the *rsm1* gene is really synthetic lethal with *spmex67*, we crossed the  $\Delta rsm1$  mutant strain with the  $\Delta spmex67$  mutant strain. To allow growth in the synthetic lethal background, the double mutant cells also carried a plasmid that expresses *spmex67* from the weak thiamine-repressible *nmt1* promoter on the pREP81X vector. As shown in Fig. 3, the single mutants grew well. However, the combination of the  $\Delta rsm1$  mutation with  $\Delta spmex67$  mutation resulted in synthetic lethality when the expression of *spmex67* in the double mutant was repressed by the addition of thiamine. This result confirmed that *rsm1* is genetically linked with *spmex67*.

#### Synthetic lethality induced an mRNA export defect

The viability of the  $\Delta rsm1$  mutant suggested that Rsm1p might have an accessory role in mRNA export of *S. pombe*. To determine whether the *rsm1* gene is involved in mRNA export, poly(A)<sup>+</sup> RNA distribution was examined in the  $\Delta rsm1$  mutants. The poly(A)<sup>+</sup> RNA in the wild



**Fig. 3.** *rsm1* null allele is synthetic lethal with the *spmex67* null allele. Growths of  $\Delta rsm1$  mutant and  $\Delta spmex67$  mutant were compared to that of  $\Delta rsm1\Delta spmex67$  synthetic lethal mutants carrying pREP81X-mex67 as indicated. Cells were streaked onto EMM agar in the absence (-B1) and presence (+B1) of thiamine, and were incubated for 4 days at 28°C.



**Fig. 4.** Poly(A)<sup>+</sup> RNA localization in the  $\Delta rsm1$  mutants and  $\Delta rsm1\Delta spmex67$  synthetic lethal mutants carrying pREP81X-mex67. Cells were grown to the mid-log phase in appropriately supplemented EMM medium in the absence of thiamine (-B1) at 28°C. The cells were then shifted to EMM medium containing thiamine (+B1) and were grown for 18 h. Coincident DAPI staining is shown in the right panels.

type strain (*rsm1*<sup>+</sup>) and  $\Delta spmex67$  mutant is distributed throughout the whole cell (Yoon *et al.*, 2000). However, poly(A)<sup>+</sup> RNA in  $\Delta rsm1$  mutants was accumulated slightly more in the nucleus (Fig. 4). This result suggests that Rsm1p may have an accessory role in mRNA export from the nucleus, though it is not essential for bulk poly(A)<sup>+</sup> RNA export.

Next, to determine whether the growth defect of the synthetic lethal mutations was associated with mRNA export defects, poly(A)<sup>+</sup> RNA distribution was examined in  $\Delta rsm1\Delta spmex67$  double mutants grown under permissive and restrictive conditions. The  $\Delta spmex67$  strain carrying pREP81X-*spmex67*<sup>+</sup>, had no observable mRNA export defect, whether grown in the presence or absence of thiamine (Yoon *et al.*, 2000). In the case of  $\Delta rsm1$

$\Delta spmex67$  double mutants, when *spmex67*<sup>+</sup> was expressed from the thiamine-repressible *nmt81* promoter in the absence of thiamine (-B1), poly(A)<sup>+</sup> RNA was distributed throughout the whole cell, with a slight accumulation in the nucleus like in the  $\Delta rsm1$  mutant cells. This demonstrated that the mutant cells had only minor mRNA export defects when grown in permissive conditions. However, after repression of *spmex67*<sup>+</sup> for 12 h by growing synthetic lethal mutants in the presence of thiamine (in synthetic lethal condition), most cells showed extensive poly(A)<sup>+</sup> RNA accumulation in the nucleus and a decrease of poly(A)<sup>+</sup> RNA in the cytoplasm (Fig. 4). These results suggested that *spmex67* and *rsm1* genetically interact to affect the export of poly(A)<sup>+</sup> RNA from the nucleus.

In summary, we have isolated the *rsm1* gene and showed that a deletion mutation of this gene is synthetically lethal when combined with the *spmex67* null mutation, which causes a severe mRNA export defect. Also,  $\Delta rsm1$  deletion mutants showed slight mRNA export defects. Because Mex67/TAP plays a central role in mRNA export, to figure out the functions of Rsm1p in mRNA export would be interesting and important for the advancement of our knowledge about Mex67/TAP function. Further works of this type will expand our understanding of the mechanisms of the mRNA export pathway and the roles of individual proteins that are involved in mRNA export.

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