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Synthetic Lethal Mutations with *spmex67* of *Schizosaccharomyces pombe* in the Mediation of mRNA Export

Jin Ho Yoon

Department of Biology, Sungshin Women's University, Seoul 136-742, Korea

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Mex67p/Tap are evolutionally conserved mRNA export factors. To identify mutations in genes that are functionally linked to *mex67* with respect to mRNA export, we used a synthetic lethal genetic screen in *Schizosaccharomyces pombe*. Three synthetic lethal mutants were isolated and mutations in these mutants defined separate complementation groups. These mutants exhibited the accumulation of poly(A)$^+$ RNA in the nucleus, with a decrease in the cytoplasm under synthetically lethal conditions, suggesting that the mutations cause an mRNA nuclear export defect. In addition, the *S. pombe* genes that were found to be involved in mRNA export did not suppress the synthetic lethality of these mutants. These results indicate that the isolated mutants contain mutations in new genes, which are involved in mRNA export from the nucleus.

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

In eukaryotes, transport of macromolecules in and out of the nucleus occurs through the nuclear pore complex (NPC), which is embedded in the nuclear envelope that separates the nucleus from the cytoplasmic compartments. The yeast NPCs are large macromolecular assemblies of about 60 MDa in mass, which are made up of about 30 different proteins termed nucleoporins (Rout and Aitchison, 2001; Vasu and Forbes, 2001). The NPC structure consists of a core cylinder with an 8-fold rotational symmetry of spokes, a central transporter, eight cytoplasmic filaments and eight nuclear filaments intersecting at a distal ring, called the nuclear basket (Stoffler *et al.*, 1999). Nucleocytoplasmic transport events of a large number of diverse RNA and proteins through the NPCs are mediated by soluble transport receptors that specifically bind their cargoes via specific signals designated nuclear localization signals (NLSs) and nuclear export signals (NESs). 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 small GTPase Ran (Lei and Silver, 2002; Weis, 2002; 2003).

The export of mRNA is significantly more complicated than that of protein and other RNAs. Mature mRNAs are exported out of the nucleus as ribonucleoprotein (RNP) complexes. Concurrent with transcription, the coordinated assembly of export-competent RNP complexes takes place in the nucleus by the association of proteins with maturing transcripts. It has become apparent that mRNA export is tightly coupled to several steps of gene expression including transcriptional elongation, splicing, and polyadenylation (Zenklusen and Stutz, 2001; Dreyfuss *et al.*, 2002; Maniatis and Reed, 2002; Reed and Hurt, 2002; Weis, 2002). In the cytoplasm, the RNP complexes are disassembled to release mRNA and soluble export factors, and the latter are recycled back to the nucleus for additional rounds of mRNA export. Genetic and biochemical studies have identified many proteins potentially involved in mRNA export in yeast, mammalian, and viral systems (Stutz and Rosbash, 1998; Nakielny and Dreyfuss, 1999). These export factors include heterogeneous RNA associated nuclear proteins (such as hnRNP A1, hnRNP K and Npl3p), RNA export factor (REF) (such as Yra1p/ALY), splicing-related factors (such as Sub2p/UAP56), exon-junction proteins (such as Y14), putative mRNA export receptors (such as Rev, Mex67p/TAP), SR proteins, DEAD-box helicase (such as Dbp5), other conserved mRNA export factors (such as Gle1p and Gle2p/Rae1p), and components of the NPC (Lei and Silver, 2002; Reed and Hurt, 2002; Weis, 2002).

The best candidate mRNA export receptor is a heterodimer of a large subunit, Mex67, and a small subunit, Mtr2, in yeast, whose conserved metazoan counterparts are known as TAP/NXF and p15/NXT. Mex67 was first detected in *Saccharomyces cerevisiae* by a synthetic lethal screen with Nup85, a nucleoporin that functions in mRNA export (Segref *et al.*, 1997). *S. cerevisiae* Mex67p (scMex67p) is essential for growth and its temperature sensitive (ts) mutants accumulate poly(A)$^+$ RNA in the nucleus at nonpermissive temperatures. scMex67p binds

* To whom correspondence should be addressed.
(Tel) 82-2-920-7486; (Fax) 82-2-927-5565
(E-mail) jhoyoon@sungshin.ac.kr

to poly(A)$^+$ RNA, interacts with REF protein, Yra1p, and associates with the GLFG regions of Nup116p and Nup100p, to promote nuclear mRNA export (Strasser and Hurt, 2000; Strasser *et al.*, 2000; Strawn *et al.*, 2001). Mex67/TAP does not belong to the importin-β receptor family and does not appear to require a Ran system for binding and release of its cargo, mRNA. However, Mex67/TAP, like other nuclear transport receptors, shuttles between the cytoplasm and the nucleus, and interacts with FG repeat-containing nucleoporins (Conti and Izaurralde, 2001; Reed and Magni, 2001).

In *S. pombe*, Rae1p, but not Mex67p (spMex67p), is essential for growth and mRNA export (Brown *et al.*, 1995; Yoon *et al.*, 2000). A *rae1-167* ts mutant rapidly accumulates poly(A)$^+$ RNA in the nucleus at nonpermissive temperatures. Rae1p is an NPC-associated, conserved WD-domain protein that is also required for cell-cycle progression at the G_2/M boundary. Human Rae1p has been shown to bind mRNA and to shuttle between the nucleus and the cytoplasm (Pritchard *et al.*, 1999). In contrast to *S. cerevisae MEX67*, *S. pombe mex67* (*spmex67*) is not essential, and its involvement in mRNA export is revealed only by its synthetic lethality with a mutation of *rae1*. However, spMex67p also binds mRNA, shuttles between the nucleus and the cytoplasm, and functions to export mRNA. Moreover, *spmex67* expressed from 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 this work, we describe the isolation of synthetic lethal mutations with *spmex67* null allele. In addition, these mutations were shown to cause defective mRNA export and to reside in new genes involved in 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 AY217 (*h⁻ leu1-32 ura4-d18*), AY216 (*h⁺ leu1-32 ura4-d18*), and Δspmex67 (*h⁻ leu1-32 ura4-d18 Δspmex67::kan* /pREP81X-*spmex67*$^+$) (Yoon *et al.*, 2000). 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).

Synthetic lethal screen

Δspmex67 with pREP81X-*spmex67*$^+$ cells (1×10^8 cells) was mutagenized with 300 μg/ml of nitrosoguanidine (NG) for 1 h, with a 30% survival rate. After washing three times with a 0.9% solution of sodium chloride, cells were grown for 3 h in 1 ml of EMM medium without leucine. The cells were then plated onto EMM agar plates without leucine and grown at 28°C. Colonies were replica-plated onto EMM agar plates without leucine containing phloxin B in the presence (*spmex67* repressed) or absence (*spmex67* expressed) of thiamine (10 μM) and grown at 28°C for 3 days. Synthetic lethal mutants were identified as those colonies that turned red in the presence of thiamine but remained pink in the absence of thiamine. Synthetic lethal mutants were confirmed on EMM plates with and without thiamine.

In situ hybridization

In situ hybridization was performed as described previously (Amberg *et al.*, 1992) with the following modifications. Oligo-$(dT)_{50}$ carrying an α-digoxygenin at the 3' end was used as the hybridization probe. FITC-anti-digoxygenin Fab antibody (Roche Applied Science, Germany) was used for detecting the hybridization probe by fluorescence microscopy. 4, 6-Diamidino-2-phenyindole (DAPI) was used for observing DNA.

Complementation test

For the complementation test of synthetic lethal mutants with the *gle1, rae1, crp79, npp106, nup184,* and *elf1* genes, we used plasmids in which genomic DNA fragments spanning these genes were cloned into pDW232 vector. Each of the SLMex1, SLMex2, and SLMex3 mutants were transformed with pDW232 (empty vector with *ura4*), pMex67, pGle1, pRae1, pCrp79, pNpp106, pNup184, or pElf1 plasmids (Brown *et al.*, 1995; Yoon *et al.*, 1997; 2000; Whalen *et al.*, 1999; Thakurta *et al.*, 2002; Kozac *et al.*, 2002). The transformants were spread on EMM plates without uracil in the presence or absence of thiamine, incubated for 4 days at 28C, and the growth of transformants was compared.

Results and Discussion

Screen of synthetic lethal mutations with Δspmex67 allele in S. pombe

Synthetic lethality is one of the most successful genetic approaches in yeast for dissecting a macromolecular complex such as the NPC (Fabre and Hurt, 1997). The principle of a synthetic lethal screen is explained in Fig. 1A. Synthetic lethality is described as the combining of a mutation in a single gene that does not cause cell death, with a mutation in another related gene to cell death. Synthetic lethality thus should identify both physically interacting components as well as functionally overlapping components that do not physically interact with each other (Doye and Hurt 1995). Overlapping functions can be envisaged either as redundant functions or as functions that depend on each other.

A synthetic lethal screening was performed to identify

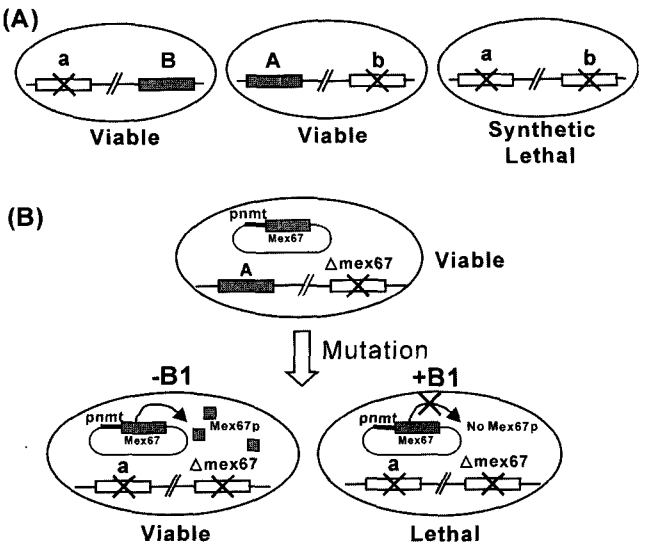

Fig. 1. (A) Schematic diagram of synthetic lethality. Wild type genes are denoted by shaded boxes and upper case letters, and mutated genes are shown by open boxes with a cross and lower case letters. (B) Schematic diagram representing synthetic lethal screening with *spmex67* null allele in *S. pombe*. Synthetic lethal mutants were kept viable by the expression of the *spmex67* gene from a pREP81X plasmid under the control of a weak, thiamine-repressible, *nmt* promoter (pnmt) in the absence of thiamine (-B1). In the presence of thiamine (+B1), the expression of *spmex67* is repressed, resulting in an inhibition of growth.

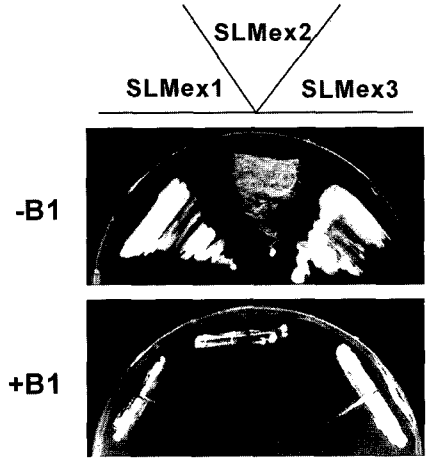

Fig. 2. Isolation of synthetic lethal mutants with the *spmex67* null allele. Synthetic lethal mutants were streaked onto EMM agar in the absence (-B1) and presence (+B1) of thiamine, and incubated for 4 days at 28°C.

genes that genetically interact with *spmex67* in *S. pombe*, which is believed to be an mRNA exporter. For this screen, we used the *spmex67* deletion allele, in which the entire *spmex67* gene was replaced by the *Kan*^{r} gene, which enables cells to resist the antibiotic G418 (Yoon *et al.*, 2000). To identify mutations that are lethal in combination with Δ*spmex67*, we used as a parental strain a Δspmex67 mutant that harbors the plasmid, pREP81X-*spmex67*$^+$, expressing *spmex67*$^+$ from the thiamine-repressible *nmt81* promoter in the absence of thiamine (vitamin B_1) but not in the presence of thiamine (Fig. 1B). This strain was mutagenized with NG, spread on EMM agar plates in the absence of thiamine, and replica-plated onto EMM agar plates in the presence of thiamine. Approximately 400,000 colonies were screened for an inability to grow in the presence of thiamine. The parental strain and mutants that have no synthetic lethal mutation were able to grow whether spMex67p from the plasmid was expressed or not, because the *spmex67* gene is not essential for growth (Yoon *et al.*, 2000). Identification of the synthetic lethal mutants was aided by the use of the dye phloxin B, which is taken up by dead cells, and which causes the colonies to become red (Yoon *et al.*, 1997). Potential synthetic lethal mutants were identified as tiny red colonies in the presence of thiamine (expression of spMex67p from the plasmid is repressed) and as big pink colonies on plates lacking thiamine. Ten candidates were isolated from the first screen. Among these, three mutants were finally confirmed and named SLMex1, SLMex2, and SLMex3, respectively (Fig. 2).

Synthetic lethal mutations define different complementation groups

The synthetic lethal mutations were separated and backcrossed to Δspmex67 strain carrying pREP81X-*spmex67*$^+$ in order to confirm that the synthetic lethality was due to a single locus. To separate the synthetic lethal mutations, synthetic lethal mutants were crossed with an spmex67$^+$ strain (AY216) and random spore analysis was performed. The spores gave rise to four different types of colonies, two parental types and two recombinant types. The parental type of colonies with both the Δ*spmex67:: Kan*^{r} allele and a synthetic lethal mutation showed the original synthetic lethal phenotype. The other parental type colonies were of the wild type. Recombinant colonies with only the Δ*spmex67:: Kan*^{r} allele could grow on media containing antibiotic, G418. The other recombinant type of colonies that had only a synthetic lethal mutation looked like a wild type. When the colonies with only the synthetic lethal mutation were crossed again with a Δspmex67 strain containing pREP81X-*spmex67*$^+$, we obtained spores that gave rise to colonies with synthetic lethal phenotypes identical to those of the original strains, SLMex1, SLMex2, and SLMex3 (data not shown). This demonstrated that the each synthetic lethal mutation alone did not affect the cell viability and that only in combination with Δ*spmex67* did they confer cell death, and thus showed that the synthetic lethality was due to a single locus.

To define the complementation groups, crosses among the synthetic lethal mutants were performed. If two mutations fell in same complementation group, no wild type spores would be produced. However, all crosses produced spores that did not show the synthetic lethal phenotype

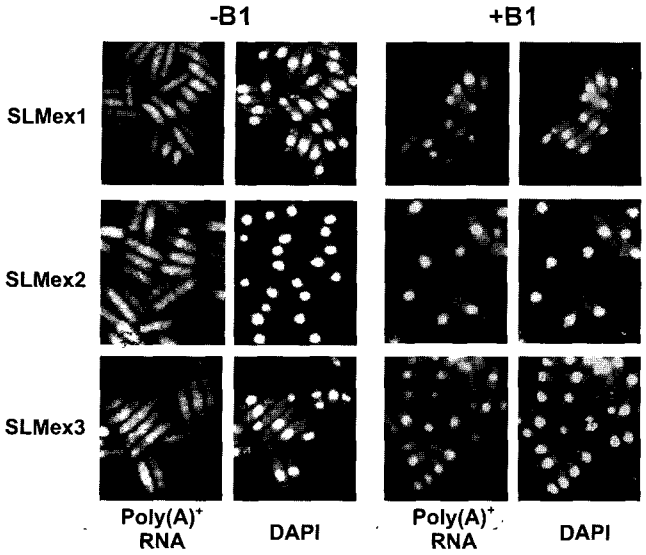

Fig. 3. Poly(A)$^+$ RNA localization in SLMex1, SLMex2, and SLMex3 synthetic lethal mutants. Cells were grown to the mid-log phase in appropriately supplemented EMM medium in the absence of thiamine (-B1) at 28°C. Cells were then shifted to EMM medium containing thiamine (+B1) and grown for 18 h. Coincident DAPI staining is shown in the right panels.

(data not shown). This result revealed that SLMex1, SLMex2, and SLMex3 fell in separate complementation groups.

The synthetic lethality induced an mRNA export defect

To determine whether the growth defect of the synthetic lethal mutations was associated with mRNA export defects, poly(A)$^+$ RNA distribution was examined in SLMex1, SLMex2, and SLMex3 mutants grown under permissive and restrictive conditions. The parental strain, Δ*spmex67* strain carrying pREP81X-*spmex67*$^+$, had no observable mRNA export defect, whether grown in the presence or absence of thiamine (Yoon *et al.*, 2000). When *spmex67*$^+$ was expressed from the thiamine-repressible *nmt81* promoter in the absence of thiamine (-B1), poly(A)$^+$ RNA in SLMex1, SLMex2, and SLMex3 mutants was distributed throughout the whole cell, with a slight accumulation in the nucleus in some cells (Fig. 3). This demonstrated that the synthetic lethal mutants had no significant mRNA export defects when grown in permissive conditions. However, after repression of *spmex67*$^+$ for 12 h by growing synthetic lethal mutants in the presence of thiamine (+B1), most cells showed extensive poly(A)$^+$ RNA accumulation in the nucleus, and a decrease in the cytoplasm (Fig. 3). These results suggest that *spmex67* and these mutated genes genetically interact to affect the export of poly(A)$^+$ RNA from the nucleus.

Synthetic lethal mutations are not linked to the S. pombe genes already known to be involved in mRNA export

In *S. pombe*, several genes have been isolated and are known to be involved in mRNA export. Temperature-sensitive mutant in the *rae1* gene was first isolated and showed mRNA export and cell cycle progression defects in restrictive temperature (Brown et al., 1995). Mutants in the *npp106*, *nup184*, and *crp79* genes were later isolated and found to be synthetically lethal with temperature-sensitive *rae1-167* (Yoon *et al.*, 1997; Whalen *et al.*, 1999; Thakurta *et al.*, 2002). In addition, the *spmex67* gene was identified as a multicopy suppressor of *rae1-167 nup184-1* synthetic lethality (Yoon *et al.*, 2000). Recently, the *elf1* gene was identified and found to be genetically linked to *rae1* and *spmex67* with respect to mRNA export (Kozak *et al.*, 2002).

Table 1. Complementation testing of synthetic lethal mutants with the genes known to be involved in mRNA export in *S. pombe*

Strain - Plasmid	SLMex1	SLMex2	SLMex3
pDW 232	–	–	–
pMex 67	+++	+++	+++
pGel 1	+	–	+
pRae 1	+	–	+
pNpp 106	–	–	–
pNup 184	–	–	–
pCrp 79	–	–	–
pElf 1	–	–	–

Since the genes mentioned above are involved in mRNA export out of nucleus and are synthetically lethal with *spmex67*, we wanted to know whether synthetic lethal mutations in the SLMex1, SLMex2, and SLMex3 mutants reside in the genes mentioned above or in new genes. For this purpose, the synthetic lethal mutants were transformed with the empty vector, pDW232, or a plasmid bearing the genomic *spmex67, gle1, rae1, crp79, npp106, nup184,* and *elf1* genes, respectively, and spread on EMM media in the presence or absence of thiamine. The synthetic lethal mutants grew well and formed large colonies in the absence of thiamine, owing to the expression of *spmex67* from the thiamine-repressible *nmt81* promoter on the pREP81X vector. As shown in Table 1, mutants transformed with pDW232 as a negative control were unable to grow under the synthetic lethal condition (i.e. in the presence of thiamine). However, the plasmid harboring genomic *spmex67*, as a positive control, completely rescued the growth defect of the mutants in the presence of thiamine, because spMex67p is expressed from the plasmid (pMex67) even though spMex67p expression from *nmt81* promoter on the pREP81X vector is repressed by the addition of thiamine. In contrast, the expressions of the *crp79, npp106, nup184,* and *elf1* genes from the transforming plasmids could not suppress the growth defect of

the mutants under the synthetic lethal condition. In addition, the *gle1* and *rae1* expressions were unable to completely restore mutant growth. This result indicates that these are not the cognate synthetic lethal gene in the SLMex1, SLMex2, and SLMex3 mutants. Interestingly, the expressions of *gle1* and *rae1* genes partially complement synthetic lethality in SLMex1 and SLMex3 mutants, suggesting that these genes have functions overlapping *spmex67* or the mutated gene in the SLMex1 and SLMex3 mutants.

In summary, we have isolated three mutations that are synthetically lethal when combined with the *spmex67* null mutation, which causes an mRNA export defect. Because these mutations seem not to reside in genes that are experimentally known to be involved in mRNA export in *S. pombe*, it would be interesting to isolate the cognate synthetic lethal genes to figure out the functions of these genes in mRNA export. This type further works will expand our understanding of mechanisms of the mRNA export pathway and the roles of individual proteins involved in mRNA export.

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