Synergistic defect in 60S ribosomal subunit assembly caused by a mutation of Rrs1p, a ribosomal protein L11-binding protein, and 3'-extension of 5S rRNA in *Saccharomyces cerevisiae*

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ABSTRACT

Rrs1p, a ribosomal protein L11-binding protein, has an essential role in biogenesis of 60S ribosomal subunits. We obtained conditionally synthetic lethal allele with the *rrs1*-5 mutation and determined that the mutation is in *REX1*, which encodes an exonuclease. The highly conserved leucine at 305 was substituted with tryptophan in *rex1-1*. The *rex1-1* allele resulted in 3'-extended 5S rRNA. Polysome analysis revealed that *rex1-1* and *rrs1-5* caused a synergistic defect in the assembly of 60S ribosomal subunits. In *vivo* and *in vitro* binding assays indicate that Rrs1p interacts with the ribosomal protein L5–5S rRNA complex. The *rrs1-5* mutation weakens the interaction between Rrs1p with both L5 and L11. These data suggest that the assembly of L5–5S rRNA on 60S ribosomal subunits coordinates with assembly of L11 via Rrs1p.

INTRODUCTION

Eukaryotic ribosomes are synthesized mainly in the nucleolus [for reviews see (1–4)]. Yeast ribosomes consist of 4 rRNAs and 78 ribosomal proteins (RPs). All four rRNAs are encoded by a 9.1 kb rDNA unit, which is tandemly repeated 100–200 times on chromosome XII. Three rRNAs (25S, 18S and 5.8S) are transcribed as a long 35S precursor by RNA polymerase I, whereas 5S rRNA is transcribed by RNA polymerase III as a 0–extended 5S rRNA. Polysome analysis revealed that 5S rRNA is transcribed by RNA polymerase III as a slightly longer precursor with the 3'-extension (12 nt in *Saccharomyces cerevisiae*). The 35S pre-rRNA associated with *trans*-acting factors and RPs forms a large ribonucleoprotein particle, which is subsequently converted into pre-40S and pre-60S ribosomal subunits by cleavage of the pre-rRNA. It has been demonstrated that a number of *trans*-acting proteins are involved in processing of pre-rRNAs and assembly of ribosomal subunits. However, little is known about how RPs are properly recruited into pre-ribosomal subunits.

Yeast cells consume a large amount of energy in ribosomal synthesis, which accounts for 80–90% of total transcription (5). Therefore, ribosome synthesis is regulated in response to environmental changes. In *S.cerevisiae*, a secretory defect leads to a significant repression of ribosome synthesis (6), suggesting coupling between plasma membrane and ribosome synthesis. *RRS1* was identified in a screen for mutations that failed to repress RP genes resulting from a secretion block (7). We demonstrated that Rrs1p is essential for growth, localized in the nucleus with enrichment into the nucleolus, and required for ribosome biogenesis, especially for maturation of 25S rRNA and the assembly of 60S ribosomal subunits (7). Rrs1p depletion leads to the accumulation of 27SB pre-rRNA, suggesting that Rrs1p is required for the processing of 27SB into mature 25S rRNA (8). We also demonstrated that normal function of Rrs1p is required for export of 60S ribosomal subunits from the nucleolus to the cytoplasm (9). Furthermore, we isolated *RPL11A* encoding ribosomal protein L11 in yeast two-hybrid screening using *RRS1* as bait [(10), for a nomenclature of RPs, see (11)]. Ribosomal protein L11 is necessary for the assembly of 60S ribosomal subunits and is localized near the top surface of the central protuberance, where the 60S subunit potentially contacts the 40S subunit (12). We proposed that Rrs1p has a role to recruit L11 to pre-60S subunits. However, it remains unclear how Rrs1p functions in assembly of 60S ribosomal subunits. In order to learn more detailed functions of Rrs1p, in this paper, we have obtained a conditionally synthetic lethal allele with the *rrs1*-5 mutation and determined that the mutation is in *REX1/RNH70/YGR276c*. The mutant cells have 3'-extended
Interestingly, the *Escherichia coli* ribosomal subunits coordinates with the assembly of L11. Our results suggest that the assembly of L5-5S rRNA on 60S ribosomal subunits coordinates with the assembly of L11. Interestingly, the *Escherichia coli* homologue of L11 is a 5S rRNA-binding protein. We propose a model for the assembly process of the 60S ribosomal subunit.

### MATERIALS AND METHODS

#### Yeast strains, media and a library

The yeast strains used in this study are listed in Table 1. The conditional *rrs1* allele, *rrs1-5*, was generated by random-PCR mutagenesis of *RRS1* (9). Strain 4795-408 (MATa ade2 ade3 leu2 ura3 his7 can1 sap3; a gift from Dr L. Hartwell) was crossed twice with W303-background strain and KM426 (MATa ade2 ade3 leu2 ura3 trp1 can1 rrs1Δ::HIS3 rrs1-5-TRP1 integrated at *RRS1* YCp50-RRS1-ADE3) was obtained as a parental strain for mutant screening. Yeast cells were grown in YPD (yeast extract, polypeptide and glucose) rich medium, synthetic complete medium containing 2% glucose (SC) or SC dropout medium, depending on the plasmid markers. A library consisting of partial Sau3A fragments of *S. cerevisiae* genomic DNA inserted into single-copy yeast vector YCp50, was provided by Dr M. D. Rose (14). Standard techniques were used for yeast manipulation (15).

#### Plasmid construction

**YCp50 (CEN URAS)-RRS1-ADE3** was constructed as follows. The 1.6 kb EcoRI–SacI fragment containing *RRS1* was cloned into the same sites of YCp50 to generate YCp50-RRS1 [pAT-35; (7)]. The 5.0 kb BamHI–SalI fragment of pDK255 (16) containing *ADE3* was cloned into the same sites of pUC19 and the 5.0 kb SacI–SalI fragment of the generated plasmid was cloned into YCp50-RRS1 to make YCp50-RRS1-ADE3. The *RRS1* fragment in pRS313 (9) was cloned as a SacI–EcoRI fragment into pRS304 to generate pRS304-RRS1. The *RRS1* fragment in pRS304 was cloned as a SacI–XhoI fragment into pRS315 (CEN LEU2) to generate pRS315-RRS1, pRS315-rrs1-5 was similarly constructed. The plasmids for two-hybrid system were constructed by PCR cloning into pBTM116 or pACT2 as described previously (10, 17). The plasmid expressing L5-myc (YCPlac22-L5-myc) was generated by PCR cloning of an SphI–SalI fragment in pRS304 was cloned as a fragment in pRS313 (9) to generate YCp50-RRS1-ADE3. The *ADE3* fragment in pRS313 was cloned as a SacI–EcoRI fragment into YCp50 to make YCp50-RRS1-ADE3. The *RRS1* fragment in pRS313 (9) was cloned as a SacI–EcoRI fragment into pRS304 to generate pRS304-RRS1. The *RRS1* fragment in pRS304 was cloned as a SacI–XhoI fragment into pRS315 (CEN LEU2) to generate pRS315-RRS1, pRS315-rrs1-5 was similarly constructed. The plasmids for two-hybrid system were constructed by PCR cloning into pBTM116 or pACT2 as described previously (10, 17).

### Table 1. Yeast strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| W303   | MATα/MATα his3-11,15/His3-11,15 ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 can1-100/can1-100 | R. Rothstein |
| W303a  | MATα his3-11,15 ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 | R. Rothstein |
| KM148  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::LEU2 pRS313-HA-RRS1 (ARS/ CEN HA-RRS1 HIS3) | (10) |
| KM421  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::HIS3 rrs1-5-TRP1 integrated at *RRS1* | This study |
| KM426  | MATα ade2-1 ade3 ura3 leu2/trp1-1 can1-100 rrs1Δ::HIS3 rrs1-5-TRP1 integrated at *RRS1* YCp50-RRS1-ADE3 | This study |
| KM427  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::LEU2 | This study |
| KM428  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::HIS3 rrs1-5-TRP1 integrated at *RRS1* | This study |
| KM429  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::LEU2 | This study |
| KM430  | MATα his3-11,15 ade2-1 ura3-1/ura3-1 leu2-3,112/trp1-1 can1-100 rrs1Δ::HIS3 rrs1-5-TRP1 integrated at *RRS1* | This study |
| L40    | MATα his3Δ200 ade2 lys2-801am trp1-901 leu2-3,112 lys2::(lexAop)·HIS3 URA3::(lexAop)·HA-RRS1 HIS3 | R. Sternglanz |

### Isolation of mutants

To obtain mutated alleles that cause synthetic lethality with the *rrs1-5* allele, 9.2 × 10^4 cells of strain KM426 containing the plasmid YCp50-RRS1-ADE3 were plated on YPD and subsequently treated with UV at 25–30 J/m^2 (viability 20–61%). Plates were incubated at 32°C for 6 days. Colonies showing a red non-segregating phenotype were isolated and checked for whether they could not grow on 5-fluoroorotic acid (5-FOA) medium at 32°C. Sixteen selected colonies were subsequently transformed with pRS315-RRS1 or pRS315-rrs1-5 to test whether pRS315-RRS1 and not pRS315-rrs1-5 could replace YCp50-RRS1-ADE3 on SC plate containing 5-FOA, and one mutant was obtained. After crossing the mutant with the *RRS1* or *rrs1-5* strain, tetrad analysis revealed that the allele is derived from single mutation of genomic DNA.

### Cloning and sequencing of the REX1 gene

The mutated allele of the chromosomal gene was isolated by PCR. DNA fragments including the open reading frame (ORF) of *REX1* were amplified by PCR using a set of primers (5′-CCGGTCTTTAAGAAATGTCAGG-3′ and 5′-AGTAGGCTTGAAGGTATAGA-3′) (9) and total chromosomal DNA that was isolated from wild-type and *m1* mutant cells. The PCR was performed in duplicate and the products of each reaction were independently sequenced.

### Gene disruption

A deletion–insertion mutation of *REX1* was constructed in the diploid W303. The *REX1* gene was subcloned into pUC19 as a 3.1 kb EcoRI–Kpn1 fragment. Ncol fragments (221–507 amino acids) were deleted from the *REX1* gene and a 2.2 kb long Smal–Hpa1 fragment containing the *LEU2* gene from YEplac315 was inserted at Smal site of the *REX1* gene.
The resulting plasmid was excised with SacI as a 4.0 kb fragment and used to transform the diploid W303 strain. Leu<sup>+</sup> transformants were obtained and correct integration of the rexl::LEU2 gene at the homologous locus was confirmed by PCR. A correct integrant was sporulated and tetrads were dissected to obtain the rexlΔ strain.

**Polysome analysis**

Yeast crude cell extracts were overlaid on top of 11 ml of a 7–47% (w/v) sucrose gradient and centrifuged for 3.4 h at 35 000 r.p.m. at 4°C in a Hitachi RPS40T rotor as described previously (18). Gradients were collected by pumping up using a peristaltic pump and monitored at 254 nm.

[methyl-<sup>3</sup>H]Methionine pulse–chase and northern blot analyses

Processing of pre-rRNA was analyzed by [methyl-<sup>3</sup>H]methionine pulse–chase as described previously (19,20). Total RNA was separated on a denaturing 8% polyacrylamide/8 M urea gel and visualized by ethidium bromide staining or transferred to a Nytran membrane by electroblotting. Northern hybridization was carried out using a <sup>32</sup>P-labeled oligonucleotide probe, 5′-GGTAGATATGCCGCAACC for 5S rRNA.

**Two-hybrid assays**

Two kinds of plasmid for production of lexA binding domain–fusion proteins and Gal4p activation domain–fusion proteins were co-transformed into yeast L40 strain cells as described previously (17). Leu<sup>+</sup> Trp<sup>+</sup> transformants were selected and 5-fold serial dilutions of the cell cultures were stamped on SC–Leu, Trp, His plates containing 1 mM 3-amino-1, 2, 4-triazole and incubated at 30°C for 3 days unless indicated.

**Immunoprecipitation, western and northern analyses**

Yeast cells were grown in SC selective media to a mid-log phase, collected by centrifugation, washed twice with ice-cold IP buffer [50 mM Tris–HCl (pH7.5), 1 mM EDTA, 10% glycerol, 30 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethane sulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml pepstatin A] and resuspended in 100 μl of IP buffer. The cells were broken with glass beads by using multi-beads shocker (Yasui Kikai) at 4°C. The homogenates were centrifuged twice at 15 000 r.p.m. for 15 min at 4°C in a micro-centrifuge rotor. The supernatants were incubated with the anti-hemagglutinin (anti-HA, 12CA5; Roche) or anti-myc (9E10; COVANCE) mouse monoclonal antibodies and protein A-Sepharose beads (Sigma) overnight (for western blotting) or for 1.5 h (for northern blotting) at 4°C and then precipitated by centrifugation. The immunoprecipitates for western blotting were washed five times with IP buffer, fractionated by SDS–PAGE, transferred onto Hybond ECL membrane (Amersham Biosciences), and probed separately with the anti-HA or anti-myc antibodies. Horseradish peroxidase-conjugate sheep anti-mouse IgG (NA934; Amersham Biosciences) was used as the secondary antibody. Signals were visualized by Enhanced Chemiluminescence (Amersham Biosciences), according to the manufacturer’s instructions. The expression of binding domain (BD)- and activation domain (AD)-fusion proteins was detected by using antilexA binding protein antibodies [anti-LexA (2–12); sc-7544; Santa Cruz] and anti-HA antibodies, respectively. RNA was prepared by phenol/chloroform extraction from the immunoprecipitates followed by ethanol precipitation, and was resolved on a denaturing gel before northern blotting.

**In vitro interaction of Rrs1p with L5**

Expression of GST and MBP fusion proteins in E.coli BL21 was induced by the addition of 0.1 mM IPTG to a culture medium. The cells were cultured for 2 h at 37°C except for GST-L5 expression, for which cells were cultured for 2 h at 25°C. GST and MBP fusion proteins were affinity-purified with glutathione–Sepharose 4B (Amersham Biosciences) and amylose resin beads (New England Biolabs), respectively, according to the manufacturer’s instructions. Protein concentration was determined by SDS–PAGE followed by Coomassie blue staining by using BSA as a control. To examine the interaction of the purified proteins in vivo, 75 pmol of GST-L5 was incubated with 75 pmol of MBP-Rrs1p immobilized on amylose resin in 150 μl of reaction mixture [20 mM Tris–HCl (pH 7.5) and 1 mM DTT] overnight at 4°C. After six washes of the resin with the buffer containing 20 mM Tris–HCl (pH 7.5), 10% glycerol, 137 mM NaCl and 1% NP-40, the associated proteins were eluted with 30 μl of elution buffer [20 mM Tris–HCl (pH 7.5), 1 mM DTT and 1% maltose] and analyzed by SDS–PAGE followed by immunoblotting with rabbit anti-GST antibodies (kindly provided by Dr A. Kikuchi) and rabbit anti-MBP antibodies (T. Okada and K. Mizuta, unpublished data). Horseradish peroxidase-conjugate donkey anti-rabbit IgG (NA934; Amersham Biosciences) was used as the secondary antibody. Signals were visualized by Enhanced Chemiluminescence (Amersham Biosciences), according to the manufacturer’s instructions.

**RESULTS**

**Isolation of a yeast mutant that exhibits a synthetic growth defect with rrs1-5**

We previously showed that RRS1 encodes a nuclear protein essential for 25S rRNA maturation and 60S ribosomal subunit assembly in S.cerevisiae (7). In order to obtain more details about the function of Rrs1p, we isolated temperature-sensitive rrs1 mutants by random-PCR mutagenesis of RRS1 (9). Here, we tried to isolate alleles that exhibit a synthetic growth defect with rrs1. We selected one of the mutated rrs1 alleles, rrs1-5, because it results in weak temperature sensitivity owing to only one amino acid substitution of L65P. The rrs1-5 cells are not able to grow at 37°C, but grow at 35.5°C on YPD medium, whereas rrs1-84 and rrs1-124, which we previously used for analysis of Rrs1p functions, exhibit severe growth defects at 35.5°C (9). In this study, we screened mutants based on plasmid dependency for growth by both a red and white colony sectoring assay and counter-selection on a medium containing 5-FOA (Figure 1A and B). Parent strain cells were mutagenized by UV irradiation to 20–61% survival. Mutations that conferred synthetic lethality with rrs1-5 at 32°C were identified as red, non-sectoring colonies on plates under non-selective conditions for the plasmid, because growth is dependent on RRS1 in the ADE3-containing plasmid. Among 9.2 × 10<sup>8</sup> colonies screened, 64 exhibited this phenotype. Among them, 16 colonies were unable to
grow on medium containing 5-FOA, a drug that kills cells harboring the *URA3* gene. To confirm the synthetic growth defect with *rrs1-5*, the *LEU2*-based plasmid containing the *RRS1* gene or the *rrs1-5* gene was transformed. In only one strain, the plasmid containing the *RRS1* gene, but not the *rrs1-5* gene could replace the *YCP50-RRS1-ADE3* plasmid at 32°C by plasmid shuffling. As the mutant strain without the *RRS1* plasmid was obtained on a plate containing 5-FOA at 25°C, it was temporarily designated as *m1* mutant. Genetic analysis revealed that a recessive mutation in the genome resulted in the recessive synthesis defect in the *m1* mutant (data not shown).

**REX1/RNH70 is mutated in the *m1* mutant**

In order to isolate a gene complementing the growth defect of the *m1* mutant, the strain was transformed with a library of yeast genomic DNA constructed in *YCP50* (14) and cultured at 33°C. Of 1.4 × 10⁴ Ura⁺ transformants, six colonies grew well at 33°C, but not at 37°C on YPD medium, which is similar to the temperature sensitivity of *rrs1-5*. One of the six plasmids that were isolated from the colonies complemented the temperature sensitivity of the *m1* mutant cells, whereas the others not. A partial DNA sequence of the plasmid revealed that the insert DNA corresponds to chromosome VII from 1037596 to 1048620, including *YGR271c-A*, *YGR272c*, *YGR273c*, *TAFL*, *RTTI02*, *REXI*, *YGR277c* and *CWCC22*. Subcloning revealed that the complementing activity was contained in *REX1/RNH70/YGR276c* (data not shown). In order to confirm that the mutation of *REXI* is responsible for the synergistic temperature sensitivity of the *m1* mutant, DNA fragments containing the *REXI* gene were amplified by PCR using chromosomal DNA extracted from wild-type and the *m1* mutant cells. The DNA fragment from wild-type cells, but not that of the *m1* mutant cells, could complement the temperature sensitivity of the *m1* mutant cells (data not shown). This result confirms that *REXI* is mutated in the *m1* mutant cells and is responsible for the synthetic defect with *rrs1-5*.

*REXI* encodes an exonuclease consisting of 553 amino acid residues. DNA sequence analysis revealed that the *m1* mutant had a point mutation in *REXI*, a T-to-G conversion within codon 305, which changed a **T**G leucine codon to a **T**G tryptophan codon. We named the allele *rex1-1*. The plasmid containing *rex1-1* constructed by site-directed mutagenesis could not complement the temperature sensitivity of the *m1* mutant cells (data not shown), confirming that *rex1-1* is responsible for the synthetic growth defect with *rrs1-5*. By searching the protein database at DDBJ using the BLAST program, we found many similar sequences from various organisms. *Schizosaccharomyces pombe* putative exonuclease (NP_594627.1) has similarity to Rex1p throughout the whole sequence. The restriction region from around (196–225) to around (377–384) amino acids in Rex1p is similar to various sequences such as *Caenorhabditis elegans* exonuclease (NP_504838.1), *Drosophila melanogaster* LD30051p (AAN71248.1), *Mus musculus* similar to RIKEN cDNA 1700021p10 (XP_14328.2) and *Homo sapiens* exonuclease GOR (NP_258439.1). An alignment of the conserved region (225–377 amino acids in Rex1p) reveals that leucine 305, the mutated amino acid in *rex1-1*, is conserved in the middle of this region (data not shown).

**REXI is not required for cell growth when *RRS1* functions normally**

After crossing the *m1* mutant with a wild-type strain, we obtained a *rex1-1 RRS1* strain. This strain grows well at 25, 33 and 37°C whereas the *rex1-1 rrs1-5* strain shows a severe temperature sensitivity (Figure 1C). As the *rex1-1* mutation exhibited synthetic sick phenotype with *rrs1-5*, we determined the effect of the *rex1-null* mutation on the synthetic phenotype. A disruptant of *REXI*, *rex1Δ* grows well at 25, 33 or 37°C similar to *rex1-1* and *rex1Δ* has a similar synthetic growth defect with *rrs1-5* (Figure 1C). Furthermore, *rex1Δ* exhibits a synthetic growth defect with other *rrs1* alleles, *rrs1-84* and *rrs1-124* (data not shown). These data indicate that *REXI* is not required for cell growth, but normal function of *REXI* is required in the background of *rrs1*.

**rex1-1 leads to extended 5S rRNA**

Previously it was reported that Rex1p has a role in maturation of 5S rRNA and that *rex1Δ* results in accumulation of 3′-extended 5S rRNA by ~3 nt (13). Consistent with this observation, the *rex1Δ* strain accumulates 5S rRNA that is
longer than the corresponding RNA in wild type (Figure 2A). The \textit{rex1-1} strain also accumulates extended 5S rRNA with the same length as in the \textit{rex1} mutant (Figure 2A). On the other hand, 5.8S rRNA is normally produced in both the \textit{rex1-1} and \textit{rex1} mutant strains (Figure 2A). In the \textit{rex1-1 rrs1-5} mutant strain, extended 5S rRNA appears to be partially trimmed compared with that in the \textit{rex1-1} and \textit{rex1} mutant strains at both 25 and 33°C (Figure 2A). On the other hand, in the \textit{rex1} mutant strain cells, such trimming is not detected, and 5S rRNA appears to have similar mobility to that in the \textit{rex1} mutant strain (Figure 2A). These results indicate that the \textit{rex1-1} strain cells have very weak activity to trim the 3'-extension of 5S rRNA, which is detected only in the background of \textit{rrs1-5}.

In order to examine whether \textit{rex1-1} affects processing of 35S pre-rRNA, we performed a [methyl-\textsuperscript{3}H]methionine pulse–chase analysis (newly synthesized pre-rRNA is methylated immediately). Figure 2B shows that \textit{rex1-1} does not cause a defect in maturation of 25S or 18S rRNA (lanes 1–6 and 13–18). This suggests that the 3'-extended 5S rRNA has little effect on processing of 35S pre-rRNA to mature RNAs. On the other hand, \textit{rrs1-5} results in a slight defect in maturation of 25S rRNA at both 33 and 25°C (Figure 2B, lanes 7–9 and 19–21). In the \textit{rex1-1 rrs1-5} mutant strain, more accumulation of the 35S and 32S pre-rRNAs and slower processing of 27S to 25S were observed at 33°C (Figure 2B, lanes 22–24) compared with the \textit{rrs1-5} mutant (Figure 2B, lanes 19–21).

\textbf{rex1-1} and \textit{rrs1-5} show a synthetic defect in ribosome biogenesis

As both \textit{Rex1p} and \textit{Rrs1p} are involved in biogenesis of 60S ribosomal subunits, we analyzed polysome profiles by sucrose
density gradient ultracentrifugation (Figure 3). Five hours after shifting the rex1-1 rrs1-5 strain cells from 25 to 33°C, 40S subunits accumulated, 60S subunits, 80S monosomes and polysomes decreased and half-mer polysomes, which contain 43S initiation complexes stalled at the AUG start codon, appeared. This result indicates that the rex1-1 rrs1-5 strain is defective in biogenesis of 60S ribosomal subunits at 33°C. Even at 25°C, half-mer polysomes appear in the rex1-1 rrs1-5 strain. However, both the rex1-1 strain and the rrs1-5 strain appear to produce 60S subunits at 25 and at 33°C. This is because rrs1-5 is a weak temperature sensitive allele, although it causes a defect in biogenesis of 60S ribosomal subunits at 37°C (data not shown). These results indicate that rex1-1 and rrs1-5 cause a synthetic defect in assembly of 60S ribosomal subunits. Disruption of REX1 exhibited similar effects on polysome profiles to that of rex1-1; the rex1Δ rrs1-5 strain was defective in biogenesis of 60S ribosomal subunits at 33°C, whereas the rex1Δ strain appears to produce 60S subunits normally (data not shown).

Rrs1p interacts with L5/5S rRNA

During the cloning of the gene that complements the temperature sensitivity of the ml mutant, RPL5 encoding ribosomal protein L5 was isolated as a weak suppressor (data not shown). The suppressor effect is assumed to be due to increased expression of L5. The ml mutant had no mutated nucleotide in the ORF of the RPL5 gene (data not shown). Since L5 is known as a 5S rRNA-binding protein, we examined whether Rrs1p interacts with L5/5S rRNA. In a yeast two-hybrid system, interaction between Rrs1p and L5 is indicated by growth on a plate without histidine containing 3-amino-1,2,4-triazole (Figure 4A) and by increased β-galactosidase activity (data not shown). To further investigate the interaction between Rrs1p and L5/5S RNA, we performed immunoprecipitation followed by western and northern analyses. As shown in Figure 4B, HA-Rrs1p co-purified with L5-myc. Northern analysis following immunoprecipitation revealed that 5S rRNA co-purified with HA-Rrs1p (Figure 4C). These results indicate that Rrs1p interacts with the L5–5S rRNA complex. To further confirm a direct interaction, we performed an in vitro pull-down experiment. GST-L5, GST, MBP-Rrs1p and MBP were produced in E.coli and purified. GST-L5 or GST was incubated with MBP-Rrs1p or MBP immobilized on amylose resin. GST-L5 was precipitated with MBP-Rrs1p but not with MBP and GST was not precipitated with MBP-Rrs1p (Figure 4D). The result indicates that Rrs1p binds directly to L5.

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The rrs1-5 mutation in Rrs1p weakens the interaction of Rrs1p with both L5 and L11

In order to test the effect of the L65P mutation of Rrs1p on L5 and L11 interactions, we created the L65P mutation in the lexA BD-Rrs1p and Gal4p AD-Rrs1p. The apparent strength of interaction was changed depending on the BD-fusion
protein and AD-fusion protein; interaction of AD-Rrs1p with BD-L5 appears to be stronger than that of BD-Rrs1p with AD-L5, whereas interaction of BD-Rrs1p and AD-L11 appears to be stronger than that of AD-Rrs1p and BD-L11. Thus, to examine the effect of L65P mutation in Rrs1p, two sets of interactions, AD-Rrs1p/BD-L5 and BD-Rrs1p/AD-L11, were determined. As shown in Figure 5A, the L65P mutation in AD-Rrs1p and BD-Rrs1p almost lost interaction with BD-L5 and AD-L11, respectively. This effect was not caused by a difference in the expression level of AD- or BD-fusion proteins; as shown in Figure 5B, the mutation of Rrs1p had little effect on the expression level of AD-Rrs1p or BD-Rrs1p and furthermore, expression level of BD-L5 in the presence of AD-Rrs1p or AD-L11 in the presence of BD-Rrs1p was not affected by the mutation of L65P in Rrs1p. The results suggest that leucine at 65 in Rrs1p is critical for Rrs1p-L5 and Rrs1p-L11 interactions.

**DISCUSSION**

We demonstrated previously that Rrs1p has important functions in 25S rRNA maturation and 60S ribosomal subunit assembly (7) and is also required for nuclear transport of 60S ribosomal subunits (9). We proposed that Rrs1p has a function to recruit L11 into the pre-60S ribosomal particle (8). In this paper, we have isolated the *rex1-1* allele that causes a synthetic growth defect with *rrs1-5*. Rex1p was first described as Rnh70p, a 70 kDa protein that co-purified with RNase H activity. However, Rex1p/Rnh70p has no sequence similarity to known RNase H proteins and *rex1/rnh70* mutants do not show reduced RNase H activity (21,22). Although Rex1p functions in various steps of RNA processing, Rex1p appears to be a unique enzyme for 5S rRNA trimming because *rex1Δ* results in extended 5S rRNA (13). 5S rRNA (5S) is transcribed as a precursor by RNA polymerase III. The 5′ end of the mature 5S rRNA corresponds to that of the primary transcript.
whereas the 3' end is processed from a pre-5S rRNA that is extended by 12 nt. However, Rex1p and Rex2p function redundantly in 5.8S rRNA maturation and Rex1p, Rex2p and Rex3p are redundant for the processing of U5 snRNA and RNA subunit of RNase P. We show that the *rex1*-1 mutation causes extended 5S rRNA similar to a *rex1*-null mutant, without any effect on 5.8S, 18S and 25S rRNA maturation.

The results indicate that the synergistic defect was caused by the *rrs1*-5 mutation and 3' extended 5S rRNA. The *rex1*-1 mutation does not exhibit a synthetic defect with the *rrs1*-5 allele at 25°C, although at this temperature the *rex1*-1 allele has a defect in pre-5S rRNA processing. This is because the *rrs1*-5 mutant is temperature sensitive and less defective at 25°C than at 33°C. In the *rex1*-1 *rrs1*-5 strain cells, but not in the *rex1Δ* *rrs1*-5 strain cells, a smearing band showing extended 5S rRNA is detected at both 25 and 33°C (Figure 2A). This suggests that in the background of *rrs1*-5, extended 5S rRNA is not assembled into the pre-ribosome particle and has a chance to be trimmed by a weak activity of Rex1 that is assembled into the pre-ribosome particle. The result suggests that normal function of Rex1p is required for the assembly of 5S rRNA into the 60S ribosomal subunit as well as for the assembly of L11. *RPL5* encoding a ribosomal protein L5 was cloned as a weak suppressor of *rrs1*-5 *rex1*-1. It was suggested that L5 protects nascent 5S rRNA and recruits 5S rRNA to ribosomal particles (23). Since it appears that extended 5S rRNA is not degraded in *rex1*-1 or *rex1Δ* strains, it is likely that excess L5 suppresses the *rex1*-1 mutation by recruiting 5S rRNA. Either *rpl11AΔ* or *rpl11bA*, which results in a decreased level of L11, did not cause a synthetic growth defect with *rrs1*-5, suggesting that a decreased level of a component of ribosome does not cause a synthetic defect with *rrs1* (T. Okada and K. Mizuta, unpublished data).

Our results suggest that the recruitment of L11 and that of L5/5S rRNA are highly coordinated and that leucine at 65 in Rrs1p is critical for Rrs1p–L5 and Rrs1p–L11 interactions. We performed tandem affinity purification and detected both L11 and L5 in the fraction purified with Rrs1p-TAP (K. Ehara and K. Mizuta, unpublished data). It remains to be elucidated if both L5/5S rRNA and L11 are recruited into pre-ribosomal particles at the same time, or if assembly of one component followed by conformational rearrangement of the particle is required for assembly of the other component.

In eukaryotes, 5S rRNA tightly binds to L5 (the eukaryotic orthologue of *E. coli* L18) and the L5–5S rRNA complex is assembled into ribosomes, whereas in bacteria, 5S rRNA is assembled into ribosomes as a complex with three RPs, L5,
L18 and L25 [for reviews see (24,25)]. In *S.cerevisiae*, both L11 (the orthologue of *E.coli* L5) and L5 (the orthologue of *E.coli* L18) are localized near the top surface of the central protuberance of the ribosomal large subunit (12,26). *E.coli* L5 homologues have similar lengths; *E.coli* L5, *H.marismortui* L5, *S.cerevisiae* L11 consists of 178, 176 and 173 amino acids, respectively. In contrast, comparison of the amino acid sequences of the *E.coli* L18 homologues reveals significant difference in their lengths; *E.coli* L18, *H.marismortui* L18, *S.cerevisiae* L5 consists of 117, 186 and 297 amino acids, respectively. In this paper, we show genetic and physical interactions among Rrs1p, L11-binding protein and L5-5S rRNA recruitment is somehow conserved in *E.coli* and *S.cerevisiae*.

It was demonstrated previously that in 5S RNA mutants, a decrease in the amount of 5S RNA was paralleled by a decrease in the amount of 60S subunits due to a specific defect in the processing of the 27SB rRNA. It was proposed that 5S RNA is recruited by pre-ribosomal particles containing the 27SB precursor and that its binding allows processing to proceed at a normal rate (27). Such a mechanism could ensure that all newly formed mature 60S subunits contain equal amounts of the three rRNAs and the RPs (27). We demonstrated previously that the depletion of Rrs1p leads to the accumulation of 27SB S and 27SB L intermediates whereas level of 27SA2 intermediate remained constant (8). The result indicates that Rrs1p is required for the processing from 27SB to 25S rRNA. These results suggest that L5–5S rRNA and L11 are recruited by Rrs1p to pre-60S subunits at the same step of the processing pathway and the recruitment is necessary for the further efficient processing.

Rrs1p is highly conserved in eukaryotes and is 36.8% identical to its human homologue (7). Several lines of evidence show that the mammalian homologues of Rrs1p, L5 and L11 play important roles in various kinds of cellular responses in higher eukaryotes: (i) The expression of *RRS1* mRNA is stimulated very early in the disease cascade in *Hdh* CAG knock-in mice, Huntington’s disease model mice (28). It was also demonstrated that *RRS1* mRNA in human brains is elevated in Huntington’s disease. (ii) Human homologues of L5 and L11 can interact with HDM2 and inhibit its function; thus, resulting in stabilization and activation of the p53 tumor suppressor, similar to ARF (29,30). (iii) L5 and L11 also interact with fragile X mental retardation 1 gene (FMR1) product. The *Drosophila* homologues of L5 and L11 were identified as components of a dFMR1-associated complex (31). (iv) *RRS1* is induced by influenza virus replication, whereas viral replication results in the downregulation of many cellular mRNAs (32).

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### Conflict of interest statement

None declared.

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