RNase MRP Cleaves Pre-tRNA$^{\text{Ser-Met}}$ in the tRNA Maturation Pathway

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Abstract

Ribonuclease mitochondrial RNA processing (RNase MRP) is a multifunctional ribonucleoprotein (RNP) complex that is involved in the maturation of various types of RNA including ribosomal RNA. RNase MRP consists of a potential catalytic RNA and several protein components, all of which are required for cell viability. We show here that the temperature-sensitive mutant of rpm1, the gene for a unique protein component of RNase MRP, accumulates the dimeric tRNA precursor, pre-tRNA$^{\text{Ser-Met}}$. To examine whether RNase MRP mediates tRNA maturation, we purified the RNase MRP holoenzyme from the fission yeast Schizosaccharomyces pombe and found that the enzyme directly and selectively cleaves pre-tRNA$^{\text{Ser-Met}}$. RNase MRP has different cellular substrates than RNase P. Whereas RNase P RNA is a catalytically active ribozyme [23–27], whereas the activity of RNase MRP RNA has not been reported [28]. RNase MRP RNA consists of two structural domains, termed Domain 1 and Domain 2 [1,2,29]. Domain 1 is believed to be a catalytic domain because the structure of this domain closely resembles that of RNase P and has major secondary structural elements conserved among RNase MRPs from a broad range of eukaryotes [29–31]. Domain 2 appears to participate in the maturation of specific tRNA in vivo. In addition, mass spectrometry–based ribonucleoproteomic analysis demonstrated that this RNase MRP consists of one RNA molecule and 11 protein components, including a previously unknown component Rpi701. Notably, limited nucleolysis of RNase MRP generated an active catalytic core consisting of partial rpm1 RNA fragments, which constitute “Domain 1” in the secondary structure of RNase MRP, and 8 proteins. Thus, the present study provides new insight into the structure and function of RNase MRP.

Introduction

Ribonuclease mitochondrial RNA processing (RNase MRP) is an essential eukaryotic ribonucleoprotein complex, generally consisting of one noncoding RNA (ncRNA) and several protein subunits [1–3]. Mutations in the human ncRNA cause a variety of recessive inherited disorders including cartilage-hair hypoplasia, which is characterized by short stature, hypoplastic hair, defective cellular immunity, and a predisposition to cancer [4–6], metaphyseal dysplasia without hypotrichosis [7], anauxetic dysplasia [8], kyphomelic dysplasia [9], and Omenn syndrome [10]. It has been reported that some inherited mutations in MRP RNA reduce the stability of the enzyme complex and/or alter its catalytic activity [11–14], but a mechanism linking the mutations to disease remains unknown.

The multisubunit composition of RNase MRP is remarkably similar to that of RNase P [1,2,15,16]. In Saccharomyces cerevisiae, RNase MRP contains a 340 nt-long RNA component and ten essential proteins (listed in Table S1), eight of which are shared with RNase P [17]. RNase MRP has two additional subunits, Snm1 and Rmp1, which are not found in RNase P [18,19]. Human RNase MRP and P also have similar subunit compositions (Table S1) [20–22].

The RNA component of RNase MRP is structurally related to that of RNase P [1,2,24]. However, the RNase P RNA is a catalytically active ribozyme [23–27], whereas the activity of RNase MRP RNA has not been reported [28]. RNase MRP RNA consists of two structural domains, termed Domain 1 and Domain 2 [1,2,29]. Domain 1 is believed to be a catalytic domain because the structure of this domain closely resembles that of RNase P and has major secondary structural elements conserved among RNase MRPs from a broad range of eukaryotes [29–31]. In addition, Domain 1 interacts with the protein subunits found in common with RNase P, including Pop1, Pop5, Pop6, Pop7, Pop8, and Rpp1 [23,32–37]. On the other hand, Domain 2 appears to determine the enzyme’s substrate specificity because the equivalent structure in RNase P serves to recognize pre-tRNA substrates [38–40]; interestingly, the Domain 2 sequence is not similar to the corresponding sequence of RNase P [1,2,24]. Although Esakova et al. recently reported that S. cerevisiae RNase MRP binds the substrate with Domains 1 and 2 in vitro [41], the structural elements that define the catalytic activity and substrate specificity of RNase MRP remain largely unknown.

RNase MRP has different cellular substrates than RNase P. Whereas RNase P cleaves primarily tRNAs and participates in tRNA maturation [3,42,43], RNase MRP targets (i) the site A$^3$ of the internal transcribed spacer 1 (ITS1) between 18S and 5.8S...
Ribosomal RNAs (rRNAs) in the precursor 27SA2 rRNA during ribosome biogenesis in the nucleolus [44,45], (ii) a subset of mRNAs involved in cell-cycle regulation [46–48], and (iii) other RNAs including a certain type of mRNA, snRNA, snoRNA, transposon RNA, and viral RNA [48–50]. It has also been reported that a dimeric tRNA precursor, pre-tRNA^Ser-Met^, might be a substrate of RNase MRP [51] because a pre-tRNA intermediate accumulates in a Schizosaccharomyces pombe mutant defective for RNase MRP. tRNA maturation requires cleavage of the dimeric pre-tRNA^Ser-Met^, which generates pre-tRNA^Ser-Met^ having a 5′ leader sequence, intron, and the 3′ “trailer” sequence, and pre-tRNA^Met^ having a mature 5′ end and 3′ trailer sequence (Figure S1) [52]. However, direct experimental evidence that RNase MRP participates in this process has not been obtained.

To elucidate the role of RNase MRP in tRNA processing, we prepared a temperature-sensitive (ts) S. pombe mutant of rpm1, a unique protein component of RNase MRP, and analyzed the phenotype of this mutant. We also purified RNase MRP from S. pombe and directly examined its catalytic activity. Based on our results, we propose that RNase MRP is responsible for the maturation of pre-tRNA^Ser-Met^.

Results

Inactivation of RNase MRP causes the accumulation of pre-tRNA^Ser-Met^.

Because all the components of RNase MRP are essential for cell viability [1,2], the cellular role of this enzyme has been studied mainly using ts mutants carrying mutations in the gene for rpm1 RNA [51–54], Rmp1 [19] or Snn1 protein [55]. We tried to isolate a fission yeast (S. pombe) ts mutant caused by mutation in Rmp1, a protein subunit specific to RNase MRP. By screening yeast strains carrying mutations in Rmp1, we obtained a ts mutant of S. pombe RNase MRP and that Domain 1, in the context of the holoenzyme, is responsible for the catalytic activity of this multisubunit enzyme complex.

RNA and protein components of S. pombe RNase MRP

Previous studies showed that the catalytically active RNase MRP isolated from yeast S. cerevisiae and from human HEp-2 cells consists of a single mrp1 RNA of 340 and 277 nt and 9 and 10 protein components, respectively [17–22]. To isolate the S. pombe RNase MRP, we employed tandem affinity purification using Rmp1 fused with a FEM-3 tag (FLAG, TEV cutting site, and 3× Myc attached to the C-terminus) as bait. The resulting complex was catalytically active against the known substrate of RNase MRP, ITS1 RNA (Figure S2). This RNase MRP preparation contained a single major RNA of ~400 nt, the predicted size of S. pombe mrp1 RNA from the size of S. cerevisiae RNase MRP RNA (Figure 2A). This RNA band was excised from the PAGE gel, digested with RNase T1 or with MfeII/PemK RNase, and the fragments were analyzed by tandem mass spectrometry (MS/MS) coupled with a genome-oriented search engine Ariadne [57]. The analysis identified all fragments covering the total sequence of mrp1 RNA (Figure 2B and Table S2). In addition, we found that the S. pombe mrp1 RNA had heterogeneous 5′-terminal sequences, AAAUG, AUG and G, each with a 5′-trimethylguanosine cap (Figure S3). This cap structure indicates that the mrp1 RNA is transcribed by RNA polymerase II, as noted for S. cerevisiae rnap1 RNA [58]. We also found that the RNA had heterogeneous 3′-terminal sequences, CUCAAG-OH and an additional one to four adenosines at the 3′ end in place of G (CUCAAA-OH, 4-OH, Figure 2B and Table S2). This supports the previous reports that the primary transcript of mrp1 is processed by an exonuclease that catalyzes 3′-truncation during the biogenesis of RNase MRP and adenosines were added later [59,60]. However, the biological significance of this heterogeneity is obscure.

The proteomic analysis of the S. pombe RNase MRP by SDS-PAGE and tandem MS identified 11 protein components (Figure 2C and Table S3, see also Nomenclature in Materials and Methods). The identified proteins included all 10 components of S. pombe RNase MRP predicted in Pombase (http://www.pombase.org/), indicating that our RNase MRP preparation was typical of those described previously. Our preparation, however, contained one additional protein subunit, Rpl701, which had not been identified in RNase MRP of any organisms studied [1–3]. Rpl701, generally known as subunit L7 of the large ribosome, was reproducibly detected in the RNase MRP complex prepared multiple times. Furthermore, the reverse pull-down analysis using a tagged Rpl701 as bait allowed isolation of RNase MRP from S. pombe, whereas tagged Rpl702 or Rpl703, the paralogs of Rpl701, failed to recover the enzyme complex (Figure S4). Thus, we concluded that Rpl701 is a novel component of RNase MRP in S. pombe. According to the image analysis of the SDS-PAGE profile, S. pombe RNase MRP complex consisted of single copies of each protein subunit, including Rpl701, except for Rpp1, which was present at two copies per complex (Table S4).

RNase MRP cleaves pre-tRNA^Ser-Met^ in vitro

To examine whether RNase MRP directly cleaves the dimeric precursor tRNA to promote tRNA maturation, we performed in vitro cleavage analysis. The purified RNase MRP pulled down with a tagged Rmp1 cleaved in vitro-transcribed pre-tRNA^Ser-Met^ into two RNA fragments under the experimental conditions employed, although it was not reactive to pre-tRNA^Ser^ used for a control RNA (Figure 3A). Kinetic analysis of this reaction estimated a Michaelis constant (K_M) of 0.112 μM and V_max of 12.9 nM/min (Figure 3B). To determine the cleavage site, we prepared a synthetic substrate, “trailer”-pre-tRNA^Met^ (Figure S1), digested it with the purified RNase MRP, and analyzed the products by SDS-PAGE.
and liquid chromatography (LC)-MS/MS. The PAGE analysis detected a single RNA product at a position corresponding to the size of mature tRNAMet (Figure 3C). The LC-MS analysis detected a nucleolytic fragment pppGGGGUAUUUUG derived from the “trailer” sequence (Figure 3D) and produced a 5′ end of mature tRNA Met. We also found that the fragment pppGGGGUAUUUUG has a hydroxyl group at 3′ terminus, consistent with the reported cleavage specificity of RNase MRP [61].

Figure 1. Pre-tRNASer-Met accumulates in the KA18 ts rmp1 mutant. (A) Rmp1 mutations in yeast strain KA18. The 11 amino acid substitutions in Rmp1 of KA18 are indicated in the figure. (B) KA18 and the control strain (KA13, Table S6) were spread onto YES plates and incubated at 30°C or 37°C for 3 days. (C) Analysis of RNAs in KA18 and KA13 cells grown at 30°C or 37°C. RNAs extracted from cells after incubation for 20 h at the indicated temperature were separated on 8 M urea-7.5% polyacrylamide gels and visualized with SYBR Gold staining. Arrows indicate RNAs that accumulated in KA18 as compared with KA13. (D) Northern blot analysis of pre-tRNASer-Met. The analysis was performed after incubation for 20 h at each indicated temperature. The srp7 RNA was used as a loading control [87,88].

The RNase-resistant core RNP of MRP cleaves pre-tRNASer-Met

To determine the structural elements necessary for the catalytic activity of RNase MRP, we performed limited nucleolysis of our RNase MRP preparation using RNase A. Although the mfp1 RNA was gradually degraded into smaller fragments by digestion with increasing RNase A concentrations at 4°C, we found two SYBR Gold-stained bands that contained relatively stable RNA fragments with approximate sizes of 150 and 120 nt (assigned as
Band 1 and Band 2 in Figure 4A). We recovered the ribonucleoprotein complex of this partial nucleolyis and examined its catalytic activity using pre-tRNA\textsubscript{Ser-Met} as a substrate. As shown in Figure 4B, this RNase A–treated MRP preparation retained the ability to cleave pre-tRNA\textsubscript{Ser-Met} (Figure 4B). Kinetic analysis estimated a \(K_{\text{M}}\) of 0.974 \(\mu\text{M}\) and \(V_{\text{max}}\) of 12.3 nM/min for the
reaction mediated by this catalytic core (Figure 4C). Although this KM value is ∼10 times greater than that estimated for the intact RNase MRP, the Vmax compares with that estimated for the intact MRP (12.9 nM/min, Figure 3B), suggesting that the limited RNase A cleavage produced an active degradation intermediate of RNase MRP with reduced affinity for the substrate RNA. We found, however, this RNase MRP intermediate did not cleave ITS1 substrate (Figure S5; see Discussion).

To characterize this partially degraded MRP complex, Bands 1 and 2 in Figure 4A were excised from a PAGE gel, in-gel digested with RNase T1, and analyzed by tandem MS; the analysis identified 24 RNA fragments for Band 1 and 18 fragments for Band 2 (Figure 4D). Mapping these fragments on the mrp1 sequence showed that they covered 100–150 nt in the 5′ and 3′ terminal regions of the mrp1 RNA. Interestingly, most of the fragments were from Domain 1 of the mrp1 secondary structure (Figure 4E). To exclude the possibility that any small RNA fragments from Domain 2 might have nucleolytic activity, we performed direct LC-MS analysis of the RNase A–treated MRP RNAs without PAGE separation. We found only a small population of RNA fragments mapped on Domain 2 (<2% of total RNA identified); (Table S5), demonstrating that the active catalytic core of RNase MRP produced by RNase A–mediated partial nucleolysis consisted of RNA fragments that are almost exclusively located in Domain 1.

We also analyzed the protein components of the active MRP core complex. The proteomic LC-MS analysis identified 8 of 11 protein subunits, whereas 3 subunits, Pop23, Rpp21, and Rpl701, were absent (Figure 4F). We estimated that the stoichiometry of the 8 subunits associated to the core complex remained essentially
the same as in the intact enzyme (Table S4), suggesting that these subunits are tightly associated with each other and with Domain 1 of the mrp1 RNA to constitute an active catalytic core of the RNase MRP complex.

Discussion

Our $K_M$ value of 112 nM for RNase MRP–mediated cleavage of tRNA<sup>Ser-Met</sup> in vitro compares well with those estimated for the catalytic reaction of tRNA precursors mediated by RNase P from various sources; i.e., 20-240 nM for RNase Ps in S. pombe [62], S. cerevisiae [63], Dictyostelium discoideum [64], and in Drosophila melanogaster [65]. In addition, it has been reported that the cellular concentration of RNase MRP is similar to that of RNase P [2] and that most RNase MRP localizes primarily in nucleoli [66,67], where pre-tRNAs exist [68]. Based on these observations, we propose that RNase MRP participates in the processing of particular pre-tRNAs in collaboration with RNase P.

Our purified RNase MRP preparation cleaved a synthetic substrate, trailer+tRNA<sup>Met</sup>, and produced a “trailer” nucleotide with 3'-OH and tRNA<sup>Met</sup> with a 5'-phosphate (Figure 3D). This is consistent with the cleavage specificity reported for RNase MRP. Regarding the sequence specificity of the cleavage, there is an argument that this enzyme cleaves at the 5'-position of the fourth nucleotide from a cytosine [69] or has a broader specificity [54]. In our experiment, the enzyme cleaved a G-U bond in a “trailer” sequence (Figure 3D), suggesting that RNase MRP has rather broad cleavage specificity that certainly requires further investigation.

Several research groups have studied RNase MRP mainly by mutational analysis of the RNA component, and the structure/function relationship of this multisubunit enzyme has been
reported [19,53–56]. In this study, we produced a core of RNase MRP by partial nucleolysis and showed its nuclease activity (Figure 4B and 4C). From the analysis of the constituents of this catalytic core, we propose that the RNP complex of Domain 1 mrp1 RNA, which associates with eight protein subunits (Popel, 5, 7, 8, and 100, Rmpl, Rpp1, and Rpp40), is responsible for the catalytic activity of RNase MRP. Another structural element, Domain 2 mrp1 RNA and three protein subunits, Pop23, Rpp21, and Rpl701, may have a role in stabilizing the enzyme substrate complex and thereby determining substrate specificity. Thus, RNase MRP has a molecular architecture similar to that of RNase P (Table S1), which is composed of a catalytically active RNA domain and a structural element important for stable binding to substrate tRNAs [70,71]. Namely, Domain 2 and its associated protein subunits in RNase P constitute a "specificity domain", which has a role in the recognition of the TCPG stem–loop of the substrate pre-tRNA and can bind to a proper position of the substrate, thus conferring the specificity for pre-tRNA substrates [38–40,72–74].

Our study identified a novel protein subunit, namely Rpl701, of fission yeast RNase MRP. Rpl701 is probably a cofactor of the Domain 2 RNP complex because it was not detected in the Domain 1–associated catalytic core (Figures 4F). Although Rpl701 is not found in S. cerevisiae or human RNase P (Table S1), recent studies identified a S. cerevisiae homolog of Rpl701 as a protein factor required to construct a proper pre-rRNP structure for accurate A 3 pre-rRNA processing [75,76]; in particular, Rpl701 is a trans-acting factor in S. cerevisiae, which potentially recruits RNase MRP to the A 3 site of rRNA or removes the enzyme from the A 3 site after the processing reaction [77]. Our observation that the RNase-resistant core of RNase MRP lacking Rpl701 did not cleave ITS1 substrate (Figure S5) also suggests that Rpl701 acts as a trans-acting factor rather than a component necessary for the catalytic activity in S. pombe RNase MRP. Thus, it might be possible that fission yeast incorporated this trans-acting factor into the functional enzyme complex during evolution, presumably to improve the efficiency of ribosome biogenesis. Regarding this point, it is interesting to note that the function of Rpl701 could not be replaced by Rpl702 or Rpl703, which has high sequence similarity to Rpl701 (87% or 55% identity, respectively).

Materials and Methods

Yeast strains, media, and culture

Table S6 lists the S. pombe strains used in this study. General genetic procedures were carried out as described [79]. Standard rich yeast extract medium supplemented with leucine (YES) and Edinburgh minimal medium were used. G418 antibiotic was purchased from Nacalai Tesque.

Nomenclature

Because the specific gene names of RNase MRP components have not been finalized for S. pombe, we defined them as in Table S1. The nomenclature was according to the sequence similarity of the protein product in S. pombe to the equivalent product in S. cerevisiae or Homo sapiens.

Construction of plasmids and transformants for tagged-protein expression

The details for the targeting and expression vectors used in this study have been archived in GenBank: pGtFEM3ki-spc323.08-kanMX6 (accession no. AB623236), containing the gene kanMX6 as a marker, was used as the targeting vector to make the JJ095 strain for purification of the MRP RNase complex. SP6 cells were transformed with the resulting vector as described [79]. To screen for kanMX6-carrying transformants, cells were spread on YES plates containing 0.1 mg/ml G418.

For constitutive expression of HATA (HA, TEV cutting site, protein A)-tagged ribosomal proteins Rpl701, Rpl702, and Rpl703 and the tag without protein, pFOX1-tp701-HATA (AB623239), pFOX1-rpl702-HATA (AB623240), pFOX1-rpl703-HATA (AB623241), and pFOX1-CHATATA (AB623238) were used as expression vectors, respectively. The JJ095 cells (Table S6) were transformed with each vector and spread on Edinburgh minimal medium plates to screen for leu2 carrying the transformants.

Random mutagenesis to establish ts rmp1 mutants

The coding DNA of rmp1 (spac323.08) containing mutations was generated by PCR amplification of S. pombe genomic DNA using primers Eco-SPAC323.08-F and Not-SPAC323.08-R (Table S7) and the nucleotide analog procedure [JBS dNTP-Mutagenesis Kit, JENA Bioscience]. The mutagenized DNA was integrated into the EcoRI-Ndel site of vector pClFLATAki-kanMX6 (AB623235). In addition, the 3’ noncoding sequence of rmp1 was amplified by PCR using primers RV-Tspac323.08-F and Sph-Tspac323.08-R (Table S7) and then integrated into the EcoRV-Sphl site of the same vector. To replace chromosomal rmp1 with a mutant allele, the plasmid was transfected into SP6 cells as described [79]. G418-resistant transformants were obtained from YES plates. To select the ts clones, the transformants were replicated onto YES plates and separately incubated at a permissive temperature (30°C) and at the nonpermissive temperature (37°C). Clones that could not grow at 37°C were considered as ts mutants for RNase MRP, and their chromosomal rmp1 DNA sequences were sequenced.

Northern blotting

Total RNA was extracted from S. pombe cells according to the method described [80]. Northern blotting was performed using a DIG RNA labeling kit (SP6/T7) and a DIG luminescent detection kit (Roche Applied Science). The template DNAs including the T7 promoter for synthesizing RNA probes to detect precursor and mature tRNAs and spf7 were amplified by PCR from S. pombe genomic DNA using the primers listed in Table S7.

Purification of the intact RNase MRP complex

Intact RNase MRP was purified as described [81] with modifications. Cells constitutively expressing FEM-3-tagged Rmp1 (JJ095) were collected from a 24 culture by centrifugation and suspended in an equal volume of lysis buffer (50 mM HEPES, pH 7.6, 300 mM potassium acetate, 5 mM magnesium acetate, 20 mM β-glycerol phosphate, 1 mM EDTA, 1 mM DTT, and homogenized using a Multi-beads shocker (Yasui Kikai Co. Ltd). After removal of the debris by centrifugation at 100,000 g for 30 min at 4°C, the extracts were incubated with anti-myc IgG (9E10) conjugated to agarose (sc-40 AC, Santa Cruz Biotechnology) at 4°C for 2 h. The precipitates were washed with wash buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% v/v NP-40) and treated with the AcTEV protease–containing buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% v/v NP-40, 1 mM DTT, and 100 U of AcTEV protease (Invitrogen) at room temperature for 1 h. After centrifugation at 10,000 g for 10 min at 4°C, each supernatant was mixed with anti-FLAG M2 agarose (50 μl, Sigma-Aldrich) for secondary purification. The mixture was incubated at 4°C for 1 h, and after washing the precipitates
with the wash buffer, RNase MRP was eluted with FLAG peptide in Tris-buffered saline (TBS: 20 mM Tris-HCl, pH 7.4, 135 mM NaCl, and 0.2 mg/ml 5× Flag peptide (Sigma-Aldrich)). The yield of the enzyme was 20 pmol from the 24 yeast culture. The RNase MRP preparation thus obtained was used directly for the RNA cleavage assay. For the component analysis, the RNase MRP preparation was used after separation of RNA and proteins via phenol–chloroform extraction [92].

Preparation of the core RNase MRP complex
To isolate the core of RNase MRP, the RNase MRP preparation on the anti-FLAG M2 agarose beads was incubated with 10 μg/ml RNase A (Sigma-Aldrich) at 4°C for 1 h. To eliminate RNase A completely from the complex, the beads were washed 10 times with vigorous agitation in 1 ml wash buffer (10 ml total, 200 volumes of the resin) before eluting the complex. The product of this partial nucleolysis was then eluted with FLAG peptide in TBS as described above and used for the in vitro RNA cleavage assay. The preparation thus obtained appeared free from RNase A activity, as the mock preparation obtained by the same procedure using RNase MRP without the tag did not cleave the RNA substrate, pre-tRNA\textsuperscript{Sec-Met}. For the analysis of RNA and protein components, the eluate was extracted with phenol–chloroform, and the resulting water phase and organic phase were subjected to LC-MS/MS for RNA and protein analysis, respectively.

In vitro RNA cleavage assay
RNA substrates were synthesized using an in vitro transcription T7 kit (Takara Bio). The DNA template for the transcription was made by PCR amplification from the S. pombe genome using the primers listed in Table S7. The RNase MRP or its core RNP complex (1 pmol) purified by immunoprecipitation was mixed with 1.25–16 pmol substrate in 20 μl digestion buffer (20 mM Tris-HCl, pH10.5, 10 mM MgCl\textsubscript{2}, 1 mM DTT, 100 mM KCl, 0.1 ng/ml BSA and 0.8 U/μl of RNasin (specific RNase inhibitor against RNases A, B, C, and placental RNase, Promega). We used this buffer solution to simulate the enzyme activity under the physiological condition, even though the activity might not be optimal for the cleavage of ITS1 sequence with respect to the overall sequence coverage of the identified peptides must exceed 40%.

RNAs were analyzed by LC-MS/MS directly without ethanol precipitation (for small RNA analysis), or after ethanol precipitation and urea-PAGE separation followed by in-gel RNase digestion (for large RNA analysis) [83]. RNases for in-gel digestion, RNase T1 (Worthington), MaxF (Takara Bio), and PemK [86] were further purified before use [82]. The resulting RNA were analyzed by a direct nanoflow LC-MS/MS system as described [82]. The mass spectrometer (Thermo Fisher Scientific) was operated in a mode to automatically switch between Orbitrap-MS and linear ion trap-MS/MS acquisition as described. We used Ariadne software [57] for database searches for RNA. The database used was the genome sequence of S. pombe [http://www. pombase.org/downloads/datasets]. The following search parameters were used: the maximum number of missed cleavages was set at 1; the variable modification parameters were two methylations per RNA fragment for any nucleotide; and an RNA mass tolerance of ±50 ppm and MS/MS tolerance of ±750 ppm were allowed.

Determination of the stoichiometry of RNase MRP
The stoichiometry of protein components in the S. pombe RNase MRP was estimated by quantitative image analysis of SDS-PAGE profiles visualized by Coomassie Brilliant Blue R-250 staining. The profiles were scanned with a GT-X900 (Epson) and quantitated by Multi Gauge ver 3.0. The method provided a linear relationship between the signal intensity and protein quantity within the range of 0.1–1.0 μg protein as estimated with human serum albumin (data not shown). The quantity of each protein was expressed relative to Rnp1, which was given a value of 1.

Affinity purification of ribosomal protein L7-associated proteins
Affinity purification of ribosomal protein L7-associated proteins (Rpl701, Rpl702, and Rpl703) was performed essentially as described under “Purification of the intact and core RNase MRP complexes” with minor modifications. Briefly, the transformants expressing the protein fused with a HATA tag were lysed, and each resulting cell lysate was incubated at 4°C for 1 h with human IgG–coupled Sepharose beads (GE Healthcare Biosciences). The beads were washed with the wash buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.25% [v/v] NP-40) and incubated with the AcTEV protease–containing buffer at room temperature for 1 h. After centrifugation at 10,000 g for 10 min at 4°C, the supernatant was analyzed by Western blotting as described above.

Supporting Information
Figure S1 Illustration of synthetic tRNA substrates and their cleavage products with RNase MRP. The synthetic tRNA mimics (pre-tRNA\textsuperscript{Sec-Met} and pre-tRNA\textsuperscript{Met}) and their cleavage products (trailer and tRNA\textsuperscript{Met}) are indicated with their names and lengths (Table S7). The length of each
component is indicated at the top of figure with an arrow. tRNA<sup>Scn</sup> includes a 16-nt intron.

(TIFF)

**Figure S2** *In vitro* cleavage assay of an RNA fragment including ITS1 using *S. pombe* RNase MRP. The purified RNase MRP was incubated with RNA including ITS1 (Table S7) at 37°C for 60 min, and the product RNAs were detected by 8 M urea-7.5% PAGE (SYBR Gold staining). Amounts (pmol) of RNase MRP and ITS1 used are indicated at the top.

(TIFF)

**Figure S3** MS/MS spectrum of the RNase T1 fragment of *mrp1* RNA with a trimethylguanosine cap. The 5′ end of the RNase T1 fragment of the *mrp1* RNA (m3GpppAAUUGp<sup>2′</sup>−3′, m/z = 1100.63) was analyzed by collision-induced dissociation. Observed fragment ions were assigned on the spectrum with an arrow (upper panel). The assigned ions were also expressed on the sequence with a bar (middle panel) and as the monoisotopic mass with red numerals (lower panel). Nomenclature of c- and y-series ions are according to Ni, J. et al. (1996) *Anal. Chem.*, 68, 1989–1999. M, parent ion; p, phosphate; B, base; m<sub>2</sub>G, trimethylguanosine.

(TIFF)

**Figure S4** Detection of the interaction between RNase MRP and three Rpl7 isoforms (Rpl701, Rpl702, Rpl703). HATA (HA, TEV cutting site, protein A)-tagged Rpl7 isoforms were expressed in J/J095 cells and pulled down with IgG-coupled Sepharose. The resulting precipitate was then analyzed by western blotting. Anti-FLAG was used to detect FEM-3-tagged Rmp1 in RNase MRP (upper panel), and anti-HA was used to detect Rpl7 isoforms (lower panel).

(TIFF)

**Figure S5** *In vitro* cleavage assay of an RNA fragment including ITS1 using RNase-resistant core MRP. The RNase-resistant core MRP or intact RNase MRP (each 1 pmol) was incubated with RNA including ITS1 (Table S7) at 37°C for 60 min, and the product RNAs were detected by 8 M urea-7.5% PAGE (SYBR Gold staining).

(TIFF)

**References**

1. Davila Lopez M, Rosenblad MA, Samuelson T (2009) Conserved and variable domains of RNase MRP RNA. RNA Biol 6: 208–220.
2. Esakova O, Krasilnikov AS (2010) Of proteins and RNA: the RNase P/MRP family. RNA 16: 1725–1747.
3. Hernandez-Cid A, Aguire-Sampieri S, Diaz-Vilchis A, Torres-Larios A (2012) Ribonucleases P/MRP and the expanding ribonucleoprotein world. IUBMB Life 64: 321–328.
4. Martin AN, Li Y (2007) RNase MRP RNA and human genetic diseases. Cell Res 17: 219–226.
5. Ridanpaa M, van Eenennaam H, Pelin K, Chadwick R, Johnson C, et al. (2001) Mutations in the RNA component of RNase MRP cause a pleiotropic human disease, cartilage-hair hypoplasia. Cell 104: 195–203.
6. Mattijssen S, Welting TJ, Pruinj GJ (2010) RNase MRP and disease. Wiley Interdiscip Rev RNA 1: 102–116.
7. Nakashima E, Mabuchi A, Kashimada K, Onishi T, Zhang J, et al. (2003) SRMRP mutations in Japanese patients with cartilage-hair hypoplasia. Am J Hum Genet A 12(3): 208–216.
8. Thiel CT, Horn D, Zabel B, Eckh A, Salinas K, et al. (2005) Severely incapacitating mutations in patients with extreme short stature identify RNA-processing endoribonuclease RMRE as an essential cell growth regulator. Am J Hum Genet 77: 795–806.
9. Kuijpers TW, Ridanpaa M, Peters M, de Boer I, Vossen JM, et al. (2003) Short-limbed dwarfism with bowing, combined immune deficiency, and late onset aplastic anaemia caused by novel mutations in the RMP1 gene. J Med Genet 40: 761–766.
10. Rosenman CM, Gu Y, Cohen A (2006) Mutations in the RNA component of RNase mitochondrial RNA processing might cause Ommen syndrome. J Allergy Clin Immun 117: 897–903.
11. Hermanns P, Bertuch AA, Bertin TK, Dawson B, Schmitt ME, et al. (2005) Consequences of mutations in the non-coding RMRP RNA in cartilage-hair hypoplasia. Hum Mol Genet 14: 3725–3740.
12. Thiel CT, Morrier G, Kaila I, Rein A, Rauch A (2007) Type and level of RMRP functional impairment predicts phenotype in the cartilage hair hypoplasia-anauxetic dysplasia spectrum. Am J Hum Genet 81: 519–529.
13. Nakashima E, Tran JR, Welting TJ, Pruinj GJ, Hirose Y, et al. (2007) Cartilage hair hypoplasia mutations that lead to RMRP promoter inefficiency or RNA transcript instability. Am J Med Genet A 143A: 2673–2681.
14. Welting TJ, Mattijssen S, Peters EM, van Doorn NL, Dekkers L, et al. (2008) Cartilage-hair hypoplasia-associated mutations in the RNase MRP P3 domain affect RNA folding and ribonucleoprotein assembly. Biochim Biophys Acta 1763: 455–466.
15. Hartmann E, Hartmann RK (2003) The enigma of ribonuclease P evolution. Trends Genet 19: 561–569.
16. Xiao S, Scott F, Ferrie CA, Englek DR (2002) Eukaryotic ribonuclease P: a plurality of ribonucleoprotein enzymes. Annu Rev Biochem 71: 165–189.
17. Chamberlain JR, Lee Y, Lane WS, Englek DR (1998) Purification and characterization of the nucleolar RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. Genes Dev 12: 1676–1690.
18. Schmitt ME, Clayton DA (1994) Characterization of a unique protein component of yeast RNase MRP: an RNA-binding protein with a zinc-cluster domain. Genes Dev 8: 2617–2628.
19. Salinas K, Wierzchicki S, Zhou L, Schmitt ME (2005) Characterization and purification of Saccharomyces cerevisiae RNase MRP reveals a new unique protein component. J Biol Chem 280: 11352–11360.

**Figure S6** SDS-PAGE profile of Rmp1-FEM3-tagged RNase MRP. The Coomassie Blue–stained bands were cut into 15 pieces (Gel 1–15) and analyzed by LC-MS/MS for protein identification as described in Materials and Methods. The results of this analysis are given in Table S3.

(TIFF)

**Table S1** Nomenclature of ribonuclease MRP complex subunits. Proteins in the same row are homologs. The components of RNase MRP and P shown here were identified in this study, predicted in Pombase, and reported by Dávila López M et al. and Esakova O et al. (RNA Biol. 2009; 6(3): 208–220. and RNA. 2010; 16(9): 1725–1747, respectively).

(XLSX)

**Table S2** Summary of MS analysis of *mrp1* RNA isolated from *S. pombe* RNase MRP.

(XLSX)

**Table S3** List of the proteins in *S. pombe* RNase MRP identified by the proteomics analysis.

(XLSX)

**Table S4** Stoichiometry in the holoenzyme and core RNase MRP complexes purified from *S. pombe*.

(XLSX)

**Table S5** The RNase A-resistant *mrp1* RNA sequence in RNase MRP identified by direct LC-MS analysis.

(XLSX)

**Table S6** *S. pombe* strains used in this study.

(XLSX)

**Table S7** Oligonucleotides used in this study.

(XLSX)

**Author Contributions**

Conceived and designed the experiments: YS JT MT TI. Performed the experiments: YS JT KA YN JK. Analyzed the data: YS JT MT TI. Contributed reagents/materials/analysis tools: KH MT TI. Wrote the paper: YS DVO MT TI.
20. Welting TJ, van Verrooijs WJ, Pruijn GJ (2004) Mutual interactions between subunits of the human RNase MRP ribonucleoprotein complex. Nucleic Acids Res 32: 2318–2346.

21. Welting TJ, Kikkert BJ, van Verrooijs WJ, Pruijn GJ (2006) Differential association of protein subunits with the human RNase MRP and RNase P complexes. RNA 12: 1373–1382.

22. Rosenblad MA, Lopez MD, Piccinelli P, Samuelson T (2006) Inventory and analysis of the protein subunits of the ribonucleases P and MRP provides further evidence of homology between the yeast and human enzymes. Nucleic Acids Res 34: 5145–5156.

23. Esakova O, Perederina A, Quan C, Berezin I, Krasilnikov AS (2008) Footprinting analysis demonstrates extensive similarity between eukaryotic RNase P and ribonucleoprotein ribonuclease MRP. RNA 14: 1558–1567.

24. Mondragon A (2013) Structural studies of RNA P. Annu Rev Biophys 42: 537–557.

25. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S (1985) The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 33: 849–857.

26. Pannucci JA, Haas ES, Hall TA, Harris JK, Brown JW (1999) RNase P RNAs from some Archaea are catalytically active. Proc Natl Acad Sci U S A 96: 7803–7808.

27. Kehreska E, Svard SG, Kirschbaum LA (2007) Eukaryotic RNA P RNA mediates cleavage in the absence of protein. Proc Natl Acad Sci U S A 104: 2062–2067.

28. Stoll LL, Clayton DA (1992) Saccharomyces cerevisiae contains an RNase MRP that cleaves at a conserved mitochondrial RNA sequence implicated in replication. Mol Cell Biol 12: 2561–2569.

29. Li X, Frank DN, Pace N, Zengel JM, Lindahl L (2002) Phylogenetic analysis of the structure of RNase MRP RNA in yeasts. RNA 8: 740–751.

30. Chen JL, Pace NR (1997) Identification of the universally conserved core of ribosomal RNA P. RNA 3: 557–560.

31. Piccinelli P, Rosenblad MA, Samuelsson T (2005) Identification and analysis of ribonuclease P and MRP RNA in a broad range of eukaryotes. Nucleic Acids Res 33: 4463–4495.

32. Pluk H, van Erpehamma R, Huisjes SA, Pruijn GJ, van Verrooijs WJ (1999) RNA(P)-protein interactions in the human RNA(P) ribonucleoprotein complex. RNA 5: 512–524.

33. Perederina A, Khaneva O, Quan C, Berezin I, Krasilnikov AS (2011) Structural organizations of yeast RNA P and RNA(P) MRP holozymes as revealed by UV-crosslinking studies of RNA-P interactions. RNA 18: 720–732.

34. Khanova E, Esakova O, Perederina A, Berezin I, Krasilnikov AS (2012) Substrate recognition by ribonucleoprotein ribonuclease MRP. RNA 18: 1565–1576.

35. Grassiet C, Lapinskii M, Vologdin A, Krasilnikov AS (2012) RNA P and 3′MRP functional core in the RNA component of RNase MRP of budding yeasts. Cell Mol Life Sci 69: 2469–2480.

36. Mathijsen S, Hinoon ER, Onneckik C, Hermanss P, Zabel B, et al. (2011) Viperin mRNA is a novel target for the human RNase MRP/RNase P endoribonuclease. Cell Mol Life Sci 68: 2469–2480.

37. Quetin LM, Schmitt ME (2001) Role of RNase P in viral RNA degradation and RNA recombination. J Virol 65: 243–253.

38. Pahl J, Clayton DA (1996) A functional dominant mutation in Schizosaccharomyces pombe RNase MRP RNA affects nuclear RNA processing and requires the mitochondrial-associated nuclear mutation pmt1-1 for viability. Nucleic Acids Res 35: 7375–7378.

39. Pahl J, Lindahl L, Archer RH, Zengel JM (2002) RNA P and RNase MRP holoenzymes. Nucleic Acids Res 30: 293–301.

40. Akai H, Hibi M, Kato S, Fujita Y, Nishioka K, et al. (2011) Intracellular localization of human RNase MRP by confocal laser scanning microscopy. J Biol Chem 286: 21318–21326.

41. Cai T, Schmitt ME, Clayton DA (1992) Mutagenesis of S. pombe, which encodes a protein component of the yeast RNA MRP, reveals a role for this ribonucleoprotein endoribonuclease in plasmid segregation. Mol Cell Biol 19: 7872–7889.

42. Marvin MC, Engelke DR (2009) RNase P: increased versatility through protein subunit evolution. Trends Biochem Sci 34: 5145–5156.

43. Levinger L, Bourne R, Kollin S, Culin E, Russell K, et al. (1998) Matrices of paired substitutions show the effects of RNA D/T loop sequence by counter-selection on canavanine. Yeast 13: 1393–1403.

44. Ararune: a database search engine for identification and chemical analysis of RNA P. Nucleic Acids Res 34: 5146–5156.

45. Green CJ, Rivera-Leon R, Vold BS (1996) The catalytic core of RNase P RNA. J Biol Chem 271: 1015–1025.

46. Li K, Smagula CS, Parsons WJ, Richardson JA, Gonzalez M, et al. (1994) Subcellular partitioning of RNA P and RNA(P) is determined by chemical analysis. J Cell Biol 124: 871–882.

47. Jacobson MR, Cao LS, Wang YL, Pederson T (1995) Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. J Cell Biol 131: 1649–1658.

48. Bertrand B, Hoauer-Scott D, Keundall A, Singh RK, Engelke DR (1998) Nucleolar localization of early tRNA processing. Genes Dev 12: 2463–2478.

49. Esakova O, Perederina A, Quan C, Berezin I, Krasilnikov AS (2011) Substrate recognition by ribonucleoprotein ribonuclease MRP. RNA 17: 356–364.

50. Goodfors RG, Coch TR (2013) 5′ terminal diversity of MRP RNA and other human noncoding RNAs revealed by deep sequencing. BMC Mol Biol 14: 23.

51. Paluh JL, Clayton DA (1996) RNA(P) ribonucleoprotein endoribonuclease cleaves at a priming site of mouse mitochondrial RNA replication. J Biol Chem 8: 409–417.

52. Drainas D, Zinmanovski S, Willis A (1989) Subcellular localization of eukaryotic RNase P. FEMS Lett 251: 81–89.

53. Khanova E, Cao LS, Wang YL, Pederson T (1995) Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. J Cell Biol 131: 1649–1658.

54. Bertrand B, Hoauer-Scott D, Keundall A, Singh RK, Engelke DR (1998) Nucleolar localization of early tRNA processing. Genes Dev 12: 2463–2478.

55. Jakovljevic J, Ohmayer U, Gamalinda M, Talkish J, Alexander L, et al. (2012) Structure and expression of Rpp20 and Rpp25. RNA 18: 1805–1822.

56. Mobley EM, Pan T (1999) Design and isolation of ribozyme-substrate pairs using RNase P-based ribozymes containing altered substrate binding sites. Nucleic Acids Res 27: 4298–4304.

57. Rossjohn J, Tiller BM, Talbert JS, Galmarina A, Talbot R, et al. (2003) Matrices of paired substitutions show the effects of RNA P/D loop sequence by counter-selection on canavanine. Yeast 19: 1649–1658.

58. Liu J, Conner BY, Zengel JM, Lindahl L (2002) Substrate specificity and complementarity of RNA P from Dictyostelium discoideum. Eur J Biochem 229: 976–980.

59. Levinger L, Bourne R, Kollin S, Culin E, Russell K, et al. (1998) Matrices of paired substitutions show the effects of RNA D/T loop sequence by counter-selection on canavanine. Yeast 13: 244–254.

60. Stathopoulos C, Kalpakli DL, Drainas D (1995) Partial purification and characterization of RNA P from Dicytostelium discoideum. Eur J Biochem 229: 976–980.

61. Esakova O, Perederina A, Quan C, Berezin I, Krasilnikov AS (2010) Subunit interaction of RNase P and RNase MRP RNA and essential for cell viability. Genes Dev 6: 1975–1985.

62. Drainas D, Zinmanovski S, Willis A (1989) Subcellular localization of eukaryotic RNase P. FEMS Lett 251: 81–89.

63. Green CJ, Rivera-Leon R, Vold BS (1996) The catalytic core of RNase P. Nucleic Acids Res 24: 1497–1503.

64. Pahl J, Clayton DA (1996) RNase MRP Cleaves Pre-rRNA
79. Suga M, Hatakeyama T (2005) A rapid and simple procedure for high-efficiency lithium acetate transformation of cryopreserved Schizosaccharomyces pombe cells. Yeast 22: 799–804.
80. Elder RT, Loh EY, Davis RW (1983) RNA from the yeast transposable element Ty1 has both ends in the direct repeats, a structure similar to retrovirus RNA. Proc Natl Acad Sci U S A 80: 2432–2436.
81. Buker SM, Iida T, Buhler M, Villen J, Gygi SP, et al. (2007) Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol 14: 200–207.
82. Taoka M, Yamauchi Y, Nobe Y, Masaki S, Nakayama H, et al. (2009) An analytical platform for mass spectrometry-based identification and chemical analysis of RNA in ribonucleoprotein complexes. Nucleic Acids Res 37: e140.
83. Lu Q, Wierzbicki S, Krashnikov AS, Schmitt ME (2010) Comparison of mitochondrial and nucleolar RNase MRP reveals identical RNA components with distinct enzymatic activities and protein components. RNA 16: 529–537.
84. Taoka M, Ichimura T, Wakamatsu-Furuta A, Kubota Y, Araki T, et al. (2003) V-1, a protein expressed transiently during murine cerebellar development, regulates actin polymerization via interaction with capping protein. J Biol Chem 278: 5864–5870.
85. Taoka M, Ikami M, Nakayama H, Masaki S, Matsuda R, et al. (2010) In-gel digestion for mass spectrometric characterization of RNA from fluorescently stained polyacrylamide gels. Anal Chem 82: 7795–7803.
86. Zhang J, Zhang Y, Zhu L, Suzuki M, Inouye M (2004) Interference of mRNA function by sequence-specific endoribonuclease PemK. J Biol Chem 279: 20676–20684.
87. Brunswald P, Liao X, Holm K, Porter G, Wise JA (1988) Identification of an essential Schizosaccharomyces pombe RNA homologous to the 7SL component of signal recognition particle. Mol Cell Biol 8: 1580–1590.
88. Ribes V, Dehoux P, Tollervey D (1988) 7SL RNA from Schizosaccharomyces pombe is encoded by a single copy essential gene. EMBO J 7: 231–237.
89. Chan PP, Lowe TM (2009) GtRNAdb: a database of transfer RNA genes detected in genomic sequence. Nucleic Acids Res 37: D93–97.