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Trm112p Is a 15-kDa Zinc Finger Protein Essential for the Activity of Two tRNA and One Protein Methyltransferases in Yeast*[S]

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The degenerate base at position 34 of the tRNA anticodon is the target of numerous modification enzymes. In *Saccharomyces cerevisiae*, five tRNAs exhibit a complex modification of uridine 34 (mcm^5U_{34} and $mcm^5s^2U_{34}$), the formation of which requires at least 25 different proteins. The addition of the last methyl group is catalyzed by the methyltransferase Trm9p. Trm9p interacts with Trm112p, a 15-kDa protein with a zinc finger domain. Trm112p is essential for the activity of Trm11p, another tRNA methyltransferase, and for Mtq2p, an enzyme that methylates the translation termination factor eRF1/Sup45. Here, we report that Trm112p is required *in vivo* for the formation of mcm^5U_{34} and $mcm^5s^2U_{34}$. When produced in *Escherichia coli*, Trm112p forms a complex with Trm9p, which renders the latter soluble. This recombinant complex catalyzes the formation of mcm^5U_{34} on tRNA *in vitro* but not $mcm^5s^2U_{34}$. An *mtq2-0 trm9-0* strain exhibits a synthetic growth defect, thus revealing the existence of an unexpected link between tRNA anticodon modification and termination of translation. Trm112p is associated with other partners involved in ribosome biogenesis and chromatin remodeling, suggesting that it has additional roles in the cell.

All RNA molecules, upon completion of their transcription, must undergo modification events collectively referred to as RNA maturation. The length of the primary transcript might be altered, by removing and/or by adding nucleotides that are not encoded by the template. Also, many chemical groups are added post-transcriptionally by specific enzymes, and certain bases are isomerized. Among all RNAs, it is tRNA that contains the broader repertoire of different modifications and also the highest proportion of modified nucleotides per molecule (1). Most tRNA modification enzymes are encoded by nonessential genes whose deletion does not detectably affect cell growth under laboratory conditions. However, certain deletions, when combined, lead to a synthetic growth defect and thus reveal the importance of the corresponding modifications (2–4). Base and ribose methylations are by far the most frequent modifications in yeast tRNA. Some methylations are catalyzed by enzymes that appear to operate alone, whereas others require auxiliary factors that have been identified in several instances such as Trm6p/Trm61p, Trm8p/82p, and Trm11p/Trm112p (2, 5, 6).

In the yeast *Saccharomyces cerevisiae*, Trm11p and Trm112p are two interacting proteins that are both required for catalyzing the formation of m^2G at position 10 in several tRNAs (2). Trm11p is the catalytic subunit, whereas the function of Trm112p, although essential for the formation of m^2G_{10}, remains elusive. Trm112p is a small 15-kDa protein that contains a zinc finger domain (2, 7). It is also required for the activity of Mtq2p, a protein methyltransferase that catalyzes the methylation of the glutamine of the universally conserved GGQ tripeptide of the translation termination factor eRF1/Sup45. In that case, it was shown that co-expression of recombinant Trm112p with Mtq2p in *Escherichia coli* leads to the production of soluble Mtq2p that otherwise is recovered in the pellet of the bacterial extract. Co-expression of Trm112p with Mtq2p is also essential to reconstitute the catalytic activity of the recombinant protein (7). A similar conclusion was reached when Trm11p and Trm112p were produced together *in vitro* in a wheat germ cell-free extract (8). Another putative partner of Trm112p is Trm9p, a tRNA MTase[3] that targets the tRNA anticodon. However, initial work on Trm9p had suggested that this enzyme could operate on its own, because some activity had been detected with a recombinant protein produced in *E. coli* (9). The fourth putative partner of Trm112p, as reported previously (10), is Lys9p, an alcohol dehydrogenase that possesses a near Rossman fold domain that lacks the seventh β-fold (2).

Trm9p catalyzes the addition of the most distal methyl group of 5-methoxycarbonylmethyluridine ($\underline{m}cm^5U$) that is found at the wobble position of $tRNA^{Arg\text{-}1}_{UCU}$ and $tRNA^{Gly\text{-}2}_{UCC}$ and of 5-methoxycarbonylmethyl-2-thiouridine ($\underline{m}cm^5s^2U$) that is present in $tRNA^{Glu\text{-}1}_{UUC}$, $tRNA^{Gln\text{-}1}_{UUG}$, and $tRNA^{Lys\text{-}2}_{UUU}$ (supplemental Fig. S1) (11). These three thiolated tRNAs are targets of a tRNase toxin produced by *Kluyveromyces lactis* and called zymocin (12, 13). Preventing the formation of either the mcm^5 or the s^2 moiety of mcm^5s^2U is sufficient to protect these tRNAs from the action of zymocin and thus render the cells resistant to that toxin. To

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[3] The abbreviations used are: MTase, methyltransferase; HPLC, high pressure liquid chromatography; RSC, remodels the structure of chromatin.

date, 11 genes are known to encode proteins required for the formation of the s^2 moiety (*NFS1*, *ISU1*, *ISU2*, *CFFD1*, *NBP35*, *CIA1*, *URM1*, *UBA4*, *NCS2(TUC2)*, *NCS6(TUC1)*, and *YOR251C*), and 14 are required for the mcm^5 moiety (*SIT4*, *SAP185*, *SAP190*, *KTI11–KTI14*, *ELP1–ELP6*, and *TRM9*) (14). Also, several zymocin-resistant mutants have been isolated that exhibit no alterations of the mcm^5s^2U formation. The effect of some of them is easily explained by an alteration of the cell wall formation that prevents the entry of the toxin such as the chitin synthase mutants *chs3* and *chs7* or the sphingolipid synthase mutant *ipt1*. For others, the reason of their resistance to the toxin is less obvious, as is the case for ribosomal proteins or several proteins involved in RNA metabolism. Interestingly, *mtq2-0* was isolated as being fully resistant to zymocin, although it shows no alteration of mcm^5s^2U formation (13, 14). In one study, it was reported that the alteration of the translation release factor eRF1/Sup45 also leads to zymocin resistance (13). eRF1 possesses the universally conserved tripeptide GGQ, the Gln of which is methylated by the complex Mtq2-Trm112p (7, 15, 16). More specifically, a substitution of GGQ by the non-methylatable sequences GGE or GGN induces strong zymocin resistance (13). This methylation is of crucial importance in translation termination, at the site of the peptidyl transfer reaction (17, 18). The link between the deficiency in Mtq2p or eRF1/Sup45 and the resistance to zymocin is yet unclear.

Genetic screens have led to the isolation of high copy suppressors of zymocin sensitivity that involve tRNA genes. First, $tRNA^{Glu}_{UUC}$ is able to rescue wild-type cell growth in the presence of zymocin (19), thus suggesting that this tRNA is a preferential target of the toxin as was then confirmed directly (12). On the other hand, overexpression of the unmodified $tRNA^{Lys}_{UUU}$ is able to suppress the defect of an *elp3-0* mutant (20, 21). It was independently reported that the alteration of eRF1 leads to a slight overexpression of several tRNA (22). Later, it was shown that this accumulation is also detected in *mtq2-0* mutants and that some *eRF1* mutants, such as the GGQ → GGE one, are also able to induce zymocin resistance. These authors concluded that the general elevation of tRNA levels in *mtq2-0* could be sufficient to explain its resistance to the drug (13, 14).

In the present study, we have addressed the role of Trm112p in mcm^5U and mcm^5s^2U formation in the yeast *S. cerevisiae*. We show that, *in vivo*, Trm112p is indispensable for the activity of Trm9p and that, *in vitro*, it is likely to form a heterodimer with Trm9p that is catalytically active, as it does with Mtq2p. Genetic analysis reveals that *trm9-0* and *mtq2-0* exhibit a synthetic growth defect. Although there are mild variations in tRNA abundance, it is unlikely that these variations are sufficient to explain the important growth defect of these cells. Also, in contrast to what was observed with other mutants (19–21), overexpression of the target tRNAs was unable to rescue these defects. Trm112p has more partners than the four originally described that are involved in tRNA biogenesis, termination of translation, and lysine biosynthesis. These new partners may reveal new functions of Trm112p and thus explain why a *trm112-0* strain is yet sicker than a strain having the quadruple deletion *lys9-0 mtq2-0 trm9-0 trm11-0*.

EXPERIMENTAL PROCEDURES

Microbiological Methods and Recombinant DNA Work—Yeast cells were grown, handled, and transformed as previously described (23, 24). The strains used in this study are listed in supplemental Table S1. Bacteria were grown in LB medium or ZYP5250 auto-induction expression medium (25). When necessary, the media were supplemented with kanamycin (35 μg/ml) and/or chloramphenicol (25 μg/ml). For expressing Trm112p, alone or with Trm9p, $ZnCl_2$ was added to a final concentration of 100 μM. The plasmids were constructed using standard recombinant techniques (26). Vector pRSFDuet-1 (Novagen) was selected because it can drive the expression of two different proteins on a single vector. The yeast *TRM112* gene with a His_6 tag on its C terminus was amplified by PCR using the plasmid pVH450 as a template (7) and primers OBL262 and OBL295 (supplemental Table S2) and inserted between the BamHI and BspHI sites of plasmid pRSFDuet-1 (MCS1) to yield plasmid pBL691. The yeast *TRM9* gene was amplified with primer OBL312 and OBL313 using genomic DNA as a template. The resulting fragment was inserted between the NdeI and XhoI sites of pBL691, thus fusing the Trm9 sequence to the S tag. High copy number plasmids expressing $tRNA^{Lys}_{UUU}$, $tRNA^{Glu}_{UUC}$, $tRNA^{Gln}_{UUG}$, or a combination of the three were previously described (20, 21).

For DNA staining, the cells were fixed in 70% ethanol containing 0.5 μg/ml of 4,6-diamidino-2-phenylindol, then rinsed in 1× phosphate-buffered saline, concentrated, and observed by fluorescence microscopy. For measurements, over 1000 cells/sample were scored for nuclei shape using Metamorph software (Molecular Devices).

For chromosome instability assay, loss of the *CAN1* and *URA3* genes was positively selected on plates containing both canavanine and 5-fluoro-orotic acid (27). A variation of the classical "gross chromosomal rearrangement" assay was used.[4] The values are calculated from at least 30 independent measurements.

RNA Preparation and Northern Hybridization—S. cerevisiae tRNA or total RNA was isolated as described (28, 29) and separated either on 8% PAGE and 8 M urea or on 1.2% agarose gel containing 1.1% formaldehyde. Northern blot was achieved on 0.45-μm charged nylon (Whatman) either for 45 min using the Panther Semidry Electroblotter (Owl) in Tris/borate/EDTA buffer (PAGE) or using a Vacu-Blot transfer apparatus (Biometra) in 10× SSC (agarose gels). The membrane was then dried at 80 °C for 30 min to immobilize tRNA. Hybridization was carried out in 6× saline/sodium phosphate/EDTA, 5× Denhardt's, and 0.5% SDS at the appropriate temperature (T_m − 5 °C). The membranes were probed with the following oligonucleotides: OBL267, $tRNA^{Ser}_{CGA}$; OBL268, $tRNA^{Met}_{iniCAU}$; OBL269, $tRNA^{His}_{GUG}$; OBL271, $tRNA^{Trp}_{CCA}$; OBL321, $tRNA^{Glu}_{UUC}$; OBL322, $tRNA^{Met}_{eloCAU}$; OBL323, $tRNA^{Gln}_{UUG}$; OBL324, $tRNA^{Lys}_{UUU}$; and OBL331, $tRNA^{Arg}_{UCU}$ (supplemental Table S2). The primers were labeled with [γ-^{33}P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) and T4 polynucleotide kinase (Invitrogen) as recommended by the manufacturer, and then unincorporated

[4] L. Dirick, manuscript in preparation.

γ-[^{33}P]ATP was removed using MicroSpin G-25 columns (GE Healthcare). Northern blots were visualized and quantified by phosphorimaging (GE Healthcare). The results were normalized with a 367-base pair probe detecting the scR1 RNA (522 nucleotides), which was amplified with primers OBL181 and OBL182, the resulting fragment being labeled with [α-^{33}P]dCTP and a random sequence octadeoxyribonucleotide kit (New England Biolabs).

Protein Expression and Purification—Trm112p was expressed, alone or with Trm9p, in strain S15 (DE3-pLysS; Stratagene) in ZYP5052 auto-induction medium (25) with appropriate antibiotics. The cells were grown overnight at 25 °C and then resuspended in buffer I (10 mM Tris-Cl, pH 8.0, 500 mM NaCl, 6 mM β-mercaptoethanol, and 10 mM $ZnCl_2$) containing an EDTA-free antiprotease mixture (Roche Applied Science) and phenylmethylsulfonyl fluoride (1 mM). The cells were broken by two successive passages through a French press (500–1000 bars). After centrifugation for 30 min at 12,000 rpm, the supernatant was loaded on a column of nickel-nitrilotriacetic acid resin (Sigma). The column was washed with buffer I, and elution of the retained proteins was achieved with buffer II (10 mM Tris-Cl, pH 8.0, 10 mM $ZnCl_2$, 250 mM imidazole, and 6 mM β-mercaptoethanol). Eluted fractions containing Trm9p/Trm112p were loaded onto a HiPrep desalting column to eliminate imidazole and concentrated in buffer III (10 mM Tris-Cl, pH 8.0, 10 mM $ZnCl_2$, and 6 mM β-mercaptoethanol). Then the concentrated fraction (~4 mg/ml) was adjusted to 50 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 10% glycerol, and 1 mg/ml bovine serum albumin fraction V and frozen in small aliquots that were kept at −20 °C.

Western Blot Analysis—Total protein extracts and soluble and insoluble proteins from *E. coli* were separated by 15% SDS-PAGE and either stained with Coomassie Blue (Sigma) or transferred onto Protran nitrocellulose (Whatman). S-tagged Trm9p was revealed with a mouse anti-S tag monoclonal antibody (1/5,000; Novagen) and His_6-tagged Trm112p with an anti-His_5 (1/1,500; Qiagen). IRdyeTM 800-conjugated anti-mouse was used as a secondary antibody (1/50,000; Thermo Scientific). The membranes were scanned and quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

Modified Nucleoside Analysis and in Vitro Methylation—The dried tRNA was dissolved in water, heated to 95 °C for 2.5 min, and quickly put on ice. A portion of it was degraded to nucleosides by nuclease P1 followed by treatment with bacterial alkaline phosphatase (30) The hydrolysate was analyzed by HPLC (31) but using a Develosil C30 reverse phase column (250 × 4.6 mm; Phenomex Ltd., Macclesfield, United Kingdom). For *in vitro* methylation assay, 100 μg of tRNA were brought to 50 mM Tris-Cl, pH 7.5, 100 mM NH_4OAc, 2 mM $MgCl_2$, 1 mM EDTA, 1% glycerol, 150 μg/ml bovine serum albumin, 10 mM dithiothreitol, and 50 μM *S*-adenosylmethionine and incubated with 1–50 μg of the recombinant enzyme for 1 h at 30 °C. tRNA was then extracted once with saturated phenol and once with a phenol/chloroform/isoamyl alcohol (50/49/1) mixture before ethanol precipitation in the presence of 0.3 M KOAc. The modified nucleosides were then analyzed by HPLC as described above.

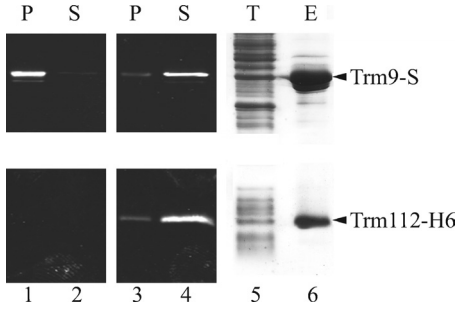

FIGURE 1. **Co-expression of Trm9p and Trm112p in *E. coli*.** Extracts were prepared from an *E. coli* strain transformed with a plasmid expressing Trm9p either alone (*lanes 1* and *2*) or with Trm112p (*lanes 3* and *4*). *Lanes 1* and *3*, insoluble pellet (*P*); *lanes 2* and *4*, soluble fraction (*S*). Western blot analysis of S-tagged Trm9p (*top panels*) and His_6-tagged Trm112p (*bottom panels*) is shown. Then a total extract (*lane 5*, *T*) was purified on a nickel-nitrilotriacetic acid column, and after extensive washes, the retained proteins were eluted with 250 mM imidazole (*lane 6*, *E*). *Lanes 5* and *6*, Coomassie Blue staining.

RESULTS

Physical Interaction between Trm112p and Trm9p—Trm112p was previously reported to interact with Trm9p, Trm11p, and Mtq2p (10). Trm112p co-precipitates with Trm11p and Mtq2q, and it improves the solubility of a recombinant Mtq2p when the two proteins are co-expressed in *E. coli* (7). To determine whether Trm112p could play a similar role on Trm9p, we prepared various constructs to express these two proteins in *E. coli*. Trm9p was expressed as a fusion with the S tag (Trm9-S), and Trm112p was expressed with a six-histidine tag (Trm112-H6), allowing their detection by Western blotting and their purification. When Trm9p is expressed without Trm112p, it is detected almost exclusively in the pellet of a cellular extract submitted to centrifugation (Fig. 1, *lane 1*). In contrast, when Trm112p is co-expressed with Trm9p in *E. coli*, most of Trm9p is detected in the soluble fraction (*lane 4*). We conclude from these observations that the ability of Trm112p to direct the proper folding of its partners is not restricted to Mtq2p and that it exerts its effect on other proteins such as Trm9p. Although this has not been strictly determined for Trm11p, there is some evidence that it is also the case for this protein. First, we had observed previously that Trm11p was mostly detected in the insoluble fraction when produced in *E. coli* and that this protein was unable to catalyze the formation of m^2G_{10} *in vitro*.[5] Second, it has been reported recently that this activity is successfully reconstituted when Trm11p and Trm112p are translated together *in vitro* in a wheat germ extract (8). Taken together, these two results strongly support the view that Trm112p is capable of directing the proper folding of Trm11p, leading to the synthesis of an active complex.

Then we tested whether recombinant Trm9-Sp could bind efficiently to recombinant Trm112-H6p by using an extract prepared from *E. coli* cells that express the two proteins. This extract was loaded onto a column containing a resin coupled to nickel-nitrilotriacetic acid, which can bond with the cyclic nitrogen atoms of two contiguous histidine residues. After extensively washing the unbound proteins, the retained proteins were eluted by gently competing with imidazole that replaces the histidine residues bound to the nickel atoms. Here,

[5] S. K. Purushothaman and B. Lapeyre, unpublished data.

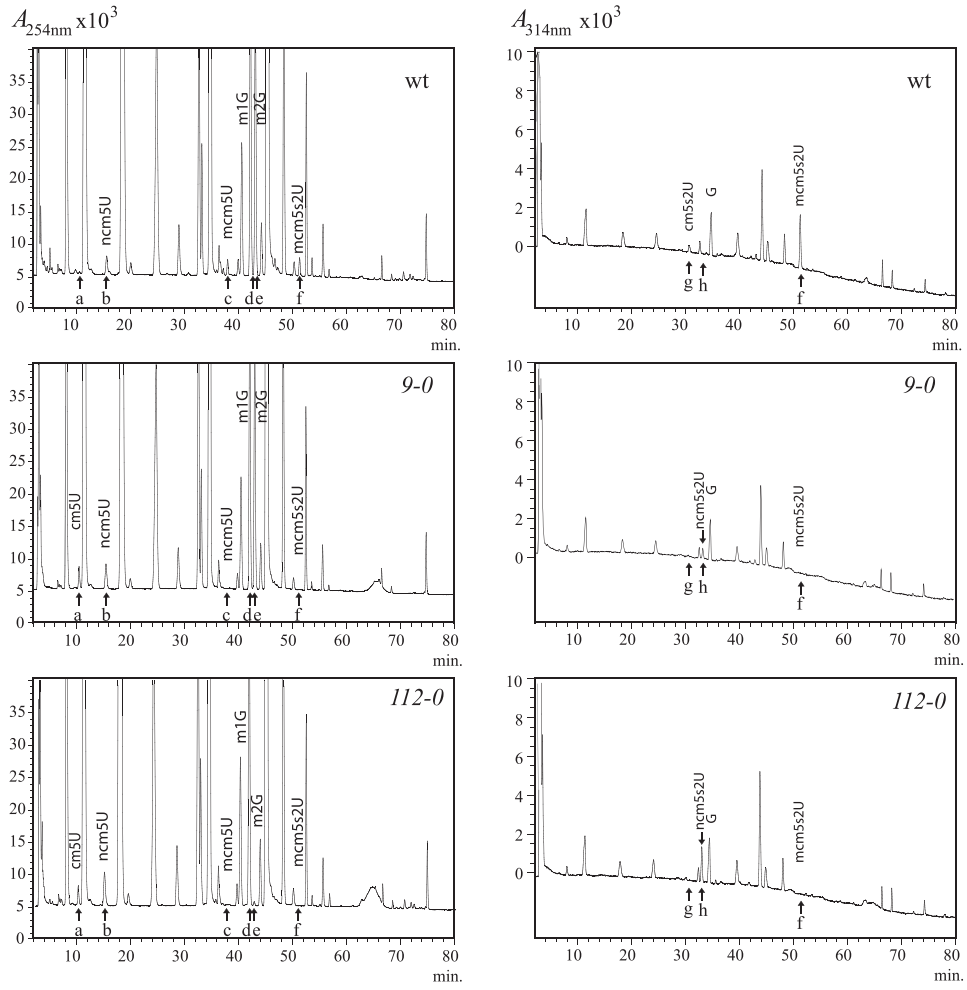

FIGURE 2. **HPLC chromatograms recorded at $A_{254\ nm}$ (*left panels*) and $A_{314\ nm}$ (*right panels*).** tRNA was degraded to nucleosides and separated on a C30 reverse phase column. The positions of relevant modified nucleosides are indicated. *Arrow a*, cm^{5}U; *arrow b*, ncm^{5}U; *arrow c*, mcm^{5}U; *arrow d*, m^{1}G; *arrow e*, m^{2}G; *arrow f*, mcm^{5}s^{2}U; *arrow g*, cm^{5}s^{2}U; *arrow h*, ncm^{5}s^{2}U. *wt*, wild type.

it is Trm112-H6p that is retained on the nickel-nitrilotriacetic acid resin because of its six-histidine tail and then released by the addition of a buffer containing 250 mM imidazole. Any protein that is bound sufficiently tightly to Trm112-H6p would then be eluted together with it. As shown here, Trm9-Sp resists extensive washes with a buffer containing 500 mM NaCl and then is co-eluted with Trm112-H6p by the addition of imidazole, thus revealing the existence of a strong interaction between these two proteins (Fig. 1, *lane 6*). It is noteworthy that the two eluted proteins appear to be in approximate stoichiometric amounts if we consider their size difference (34.2 kDa *versus* 15.9 kDa) and more importantly the differences in amino acid content; Coomassie Blue reacts with Arg, Lys, and His and with aromatic amino acids, but it is Arg that, by far, reacts the most strongly with the dye (32). Trm9-Sp contains 22 Arg *versus* only two for Trm112-H6p. These results demonstrate that recombinant Trm112-H6p and Trm9-Sp interact and form a stable and soluble complex in *E. coli*.

tRNA Modification in a trm112-0 Strain—Initially, Trm9p was identified among other candidates in a search for new potential MTases in yeast based on sequence comparison with enzymes known to use *S*-adenosyl-L-methionine as a co-factor (33). Then by using a combination of genetics and biochemical approaches, Trm9p was found to be responsible for the formation of mcm^{5}U and mcm^{5}s^{2}U in yeast tRNA (9). The authors had noticed that Trm9p was found in proteomic analyses to be associated with four partners (10, 34), and therefore they tested the requirement for three of them in mcm^{5}U formation. Neither Trm11p, nor Mtq2p, nor Lys9p were found to be required for Trm9p activity. However, the fourth candidate, Trm112p, was not tested at that time because it was categorized in yeast databases as being encoded by an essential gene, and no null mutant was available. In addition, the authors were able to detect some mcm^{5}U and mcm^{5}s^{2}U formation activity when using a recombinant fusion glutathione *S*-transferase-Trm9 protein produced in *E. coli* and saponified yeast tRNA as a substrate (9). Therefore, they concluded that Trm9p was sufficient to catalyze the last step in the formation of mcm^{5}U in yeast tRNA. However, after establishing the function of Trm11p in m^{2}G$_{10}$ formation, we then tested whether Trm112p was involved in this process. To investigate this, we obtained a diploid strain that is heterozygous for *TRM112/trm112-0*. This strain, when sporulated and dissected, gives two types of spores: two wild type and two mutants that grow very slowly (generation time, 360 ± 10 min) but are viable, nevertheless (2). This allowed us at that time to test for the effect of *trm112-0* deletion on m^{2}G$_{10}$ formation and, in the present study, on mcm^{5}U and mcm^{5}s^{2}U formation as well. Our results confirm that the formation of mcm^{5}U and mcm^{5}s^{2}U is completely abolished in the *trm9-0* strain (Fig. 2, *middle panels*), as was previously reported (9). A quantification of these compounds is presented in Table 1. Interestingly, a compound migrating after ~10 min of retention time is detected, which corresponds to an accumulation of cm^{5}U. When tRNA from a *trm112-0* strain is subjected to the same type of analysis, it clearly shows that it also lacks mcm^{5}U and mcm^{5}s^{2}U, demonstrating that, *in vivo*, Trm112p is required for the formation of these modified nucleosides to the same extent as Trm9p (Fig. 2, *bottom panels*). This phenotype is truly due to the absence of Trm112p, because a plasmid containing a wild-type copy of the *TRM112* gene (pBL652) is able to restore the formation of the two modified nucleosides in a *trm112-0* strain (Table 1). An accumulation of cm^{5}U was expected because it may be a possible intermediate in the synthesis of the mcm^5 group. However, the tRNA from both

TABLE 1
Quantification of various modified nucleosides in tRNA from wild-type and indicated mutant strains

Strain	cm^5U[a]	ncm^5U[a]	mcm^5U[a]	cm^5s^2U[b]	ncm^5s^2U[b]	m^2G[a]	mcm^5s^2U[a]
Wild-type	0.0020	0.028	0.019	0.18	ND	0.35	0.019
trm9-0	0.028	0.044	0.0029	ND	0.16	0.38	ND
trm112-0	0.018	0.041	0.00073	ND	0.49	0.0062	ND
trm11-0	0.0032	0.029	0.019	0.25	ND	0.0068	0.020
trm112-0/pBL652	0.0035	0.029	0.019	0.21	ND	0.35	0.022
mtq2-0	ND[c]	0.041	0.016	ND	ND	0.35	0.024
lys9-0	0.0032	0.029	0.020	0.22	ND	0.35	0.022

[a] The values are the absorbance at 254 nm relative to the internal standard pseudouridine.
[b] The values are the absorbance at 314 nm relative to the internal standard guanosine. For *mtq2-0* the peaks of cm^5U and cm^5s^2U were not well separated from cytidine and thus were not integrated.
[c] ND, not detected.

trm9-0 and *trm112-0* also accumulated ncm^5U and ncm^5s^2U, consistent with an earlier report for the *trm9-0* mutant (11). Whereas wild-type tRNA contains cm^5s^2U, which may be the substrate for the last methylation step in the formation of mcm^5s^2U, tRNA from the *trm9-0* and *trm112-0* mutants did not. The tRNA from *trm112-0* mutant also lacked m^2G, consistent with our previous findings that Trm112p is required for the formation of m^2G_{10} in association with Trm11p (2).

Trm9p and Trm112p Are Sufficient to Catalyze the Formation of mcm^5U in Vitro—Because we were able to produce a soluble and stable complex comprising the two recombinant proteins Trm9p and Trm112p in *E. coli*, we then tested for the ability of this complex to catalyze the formation of mcm^5U and mcm^5s^2U *in vitro*. When testing primary modification formation *in vitro*, such as m^2G_{10} formation for instance, the substrate can advantageously be a synthetic tRNA, known to be a target for this particular modification. In that case the *in vitro* transcribed tRNA bears no modification and can be readily modified by the catalytic activity to be tested. However, when testing complex modifications, such as the addition of the last methyl group of mcm^5U_{34}, the substrate must be the immediate precursor lacking this methyl group, which in the present case is supposed to be cm^5U_{34}. A tRNA containing such a modification is not easy to synthesize *in vitro*; therefore we tested two potential substrates. First, we treated total wild-type tRNA with low concentrations of NaOH, which should remove the methyl groups by saponification. In our hands, this method was rather inefficient, leading to only partial saponification and therefore compromising testing the methylation activity. Then we reasoned that, in a *trm9-0* strain, which is viable, tRNA does not contain mcm^5U nor mcm^5s^2U because of the absence of active Trm9p, but it should contain the immediate precursors, supposedly cm^5U and cm^5s^2U, which might represent good substrates to test for Trm9p/Trm112p activity. 100 μg of tRNA were treated with 1–50 μg of purified recombinant Trm9p/Trm112p, and the resulting tRNA was submitted to complete nucleoside digestion followed by HPLC analysis (Fig. 3 and Table 2). The results clearly indicate that the recombinant proteins are capable of catalyzing the formation of mcm^5U in an efficient and quantitative manner, even with low amounts of the recombinant enzyme (supplemental Fig. S2). As expected, cm^5U disappears when the tRNA is treated with the enzyme, a result that is consistent with cm^5U being the immediate precursor to mcm^5U (Table 2). However, the elevated level of ncm^5U in *trm9-0* tRNA did not decrease upon incubation with the enzyme, suggesting that it is not a substrate for it. Note also that

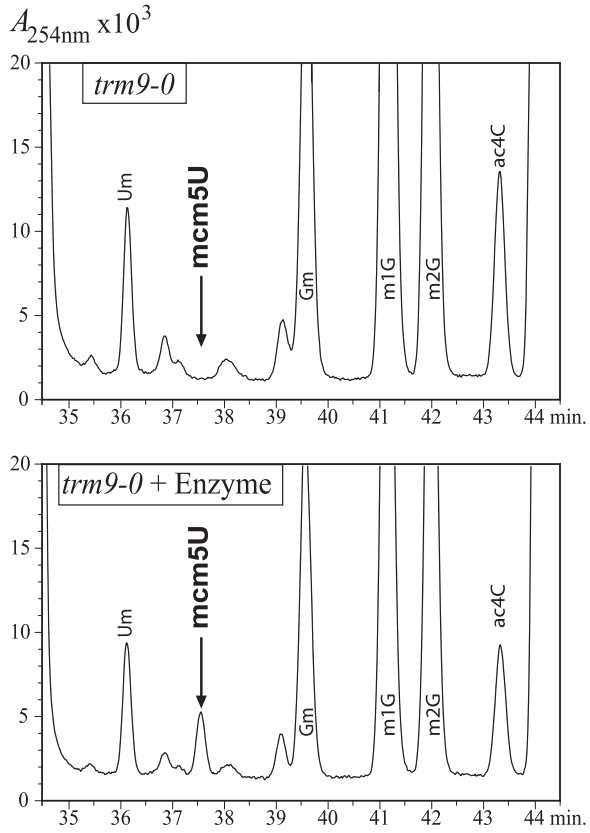

FIGURE 3. **HPLC analysis of *in vitro* modified nucleosides.** tRNA prepared from a *trm9-0* strain was treated or not with 5 μg of recombinant Trm9p/Trm112p enzyme. Then it was degraded and analyzed as in Fig. 2. The *arrow* in each panel points to the peak of mcm^5U, which is detected only after incubation with recombinant Trm9p/Trm112p (*bottom panel*).

no mcm^5s^2U was made, consistent with the observation that no cm^5s^2U was present in the *trm9-0* tRNA used as substrate (Table 2). Moreover, there was no change in the level of ncm^5s^2U upon enzyme treatment, indicating that it is not a substrate for Trm9p/Trm112p *in vitro*.

Synthetic Growth Defect of a trm9-0 mtq2-0 Double Mutant Strain—To investigate the genetic relationships existing between Trm112p and its four partners (Lys9p, Mtq2p, Trm9p, and Trm11p), we analyzed the phenotypes of the four strains in which the corresponding genes had been deleted. The four single mutant strains, *lys9-0*, *mtq2-0*, *trm9-0*, and *trm11-0*, have different growth rates; *lys9-0* and *trm11-0* are indistinguishable from the wild-type strain when grown at 30 °C in a rich medium. In contrast, the *trm9-0* strain exhibits a moderate

TABLE 2

***In vitro* methylation catalyzed by the recombinant heterodimer Trm9p/Trm112p**

The first column indicates the source of tRNA. 100 μg of tRNA were incubated with or without adding recombinant enzyme. 5 μg of recombinant enzyme (Trm9p + Trm112p) were added when indicated and incubated with *trm9-0* tRNA for 1 h at 30 °C.

tRNA	Enzyme	cm^5U[a]	ncm^5U[a]	mcm^5U[a]	cm^5s^2U[b]	ncm^5s^2U[b]	mcm^5s^2U[a]
Wild-type[c]	−	0.0020	0.028	0.020	0.18	ND	0.019
trm9-0	−	0.015	0.049	0.004	ND[d]	0.23	ND
trm9-0	+	0.001	0.047	0.024	ND	0.24	ND

[a] The values are the absorbance at 254 nm relative to the internal standard pseudouridine.
[b] The values are the absorbance at 314 nm relative to the internal standard guanosine.
[c] The data from Table 1.
[d] ND, not detected.

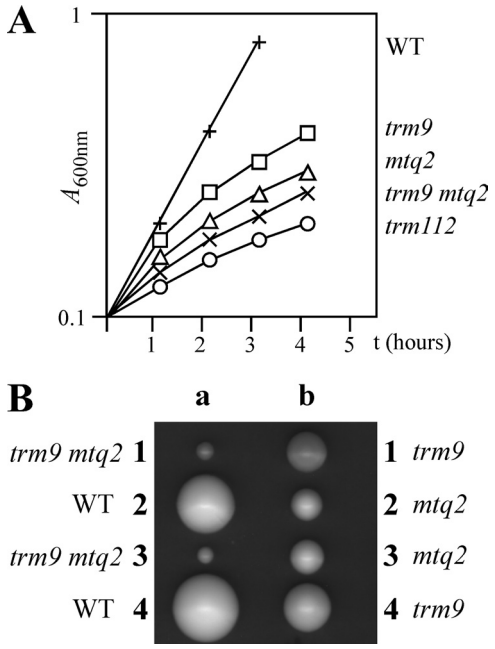

FIGURE 4. **Growth-rate comparison of wild-type and mutant strains.** A *trm9-0 mtq2-0* double mutant strain exhibits a synthetic growth defect, as compared with the two single mutant parent strains (W303 background). *A*, $A_{600\ nm}$ of various cultures grown in YPD at 30 °C were plotted at different times, as indicated. The generation time was calculated for each strain as the mean value of three independent experiments: wild type (*WT*, BMA64–1A), 90 ± 5 min; *trm9* (YBL4557), 120 ± 5 min; *mtq2* (YBL4731), 150 ± 5 min; *trm9 mtq2* (YBL4740), 210 ± 5 min; *trm112* (YBL4663), 360 ± 10 min. *B*, a diploid strain heterozygous for *trm9-0* and for *mtq2-0* was sporulated, and the tetrads thus obtained were dissected onto YPD plates that were incubated at 30 °C for various period of times. The pictures were taken, and then the colonies were tested for their genetic markers and by PCR analysis to test for the presence of the wild-type or the deleted alleles. The results for two independent tetrads (*lanes a* and *b*) are shown, the four resulting spores being shown vertically (*rows 1–4*). The results of the genotypic analysis for each spore are shown on both sides of the figure: on the *left* for tetrad a and on the *right* for tetrad b. The results are comparable with those of the liquid cultures shown above; the *mtq2-0* spores (*lane b*, *rows 2* and *3*) grew more slowly than the *trm9-0* (*lane b*, *rows 1* and *4*), which themselves grew more slowly than the wild type (*lane a*, *rows 2* and *4*). On the other hand, the two double mutant spores (*lane a*, *rows 1* and *3*) grew significantly more slowly than each single mutant parent. See also supplemental Table S3.

growth defect, and the *mtq2-0* strain exhibits a slightly more severe one (120 ± 5- and 150 ± 5 -min doubling times, respectively) (Fig. 4*A*). Then we constructed a series of 11 strains bearing all combinations of double, triple, and quadruple deletions (supplemental Table S1) and checked for any synthetic growth defect that these strains may exhibit. When the *mtq2-0* deletion was combined with the *trm9-0* deletion, a severe synthetic growth defect was observed with an extended generation time of 210 ± 5 min. When this double deletion was combined with *lys9-0*, *trm11-0*, or both, no further deterioration of the growth rate was detected (supplemental Table S3). Although one might have expected a synthetic effect when combining the deletions of activities that concur to the modification of tRNA, as was previously observed between *trm1-0* and *trm11-0*, for instance (2), it was a surprise to discover the existence of a genetic link between tRNA synthesis (Trm9p) and termination of translation (Mtq2p). Fig. 4*A* presents the growth rate of double mutant strain *mtq2-0 trm9-0* as compared with the single mutant parental strains and to a wild-type strain as a control. Interestingly, even the slowly growing *mtq2-0 trm9-0* strain grows faster that the *trm112-0* strain, suggesting that Trm112p might still have additional function(s) in the cell. On Fig. 4*B* is represented a tetrad analysis of a diploid strain heterozygous for *mtq2-0* and *trm9-0*. These two genes are not linked (chromosome IV and XIII, respectively), and therefore they segregate independently. The picture shows one tetrad that is a nonparental ditype (Fig. 4*B*, *lane a*) and a parental ditype (Fig. 4*B*, *lane b*). It is clearly visible that, on plate, the *mtq2-0 trm9-0* strain grows significantly more slowly than either the *mtq2-0* or the *trm9-0* single mutant (compare *lane a*, *row 1* with *lane b*, *rows 1* and *2*, for instance).

To better understand the genetic link existing between *TRM9* and *MTQ2*, we then investigated whether Mtq2p, in addition to its activity on eRF1/Sup45, could also be involved in tRNA modification. It was previously reported that Mtq2p was not required for mcm^5U nor mcm^5s^2U formation (14). However, it was also shown that some of the genes involved in tRNA modification behave differently in different genetic backgrounds, and there had been some conflicting reports regarding the viability or the extent of the growth defect of strains deleted for *TRM112* or *MTQ2*, for instance. Therefore, we decided to re-examine the phenotype of these mutations in different genetic backgrounds, along with the status of the tRNA modifications. tRNA was prepared from different yeast strains and subjected to complete nucleoside hydrolysis, then the resulting mixture was separated by HPLC, and the eluted nucleosides were monitored at 254 and 314 nm. A standard profile obtained with tRNA prepared from a wild-type strain is shown (Fig. 2, *top panels*), and the values obtained are represented in Table 1. No major alteration of the content of the modified nucleosides could be detected in *mtq2-0* mutant strains (Table 1), thus confirming previous observations that Mtq2p is unlikely to be required for the formation of mcm^5U and mcm^5s^2U in yeast tRNA (14).

Steady-state Levels of tRNA in trm9-0, mtq2-0, and trm112-0 Strains—tRNA modifications are involved in numerous aspects of tRNA metabolism, including controlling their stability.

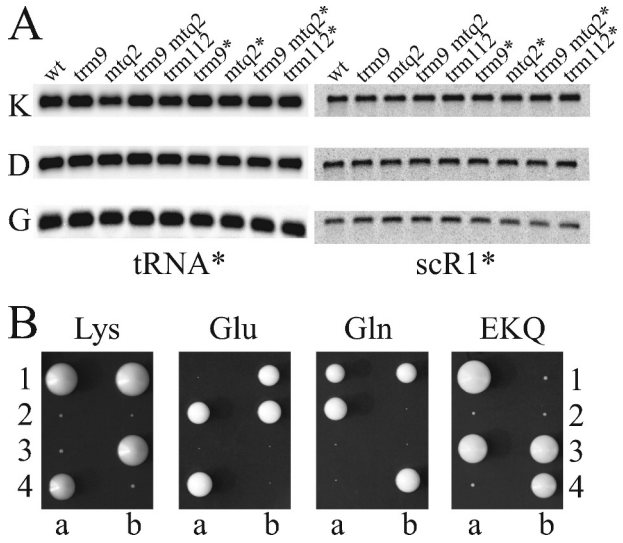

FIGURE 5. **tRNA steady-state levels in different mutants.** *A*, total RNA was separated on denaturing 1.2% agarose gel, transferred under vacuum to charged nylon membrane, and probed as indicated. *K*, *D*, and *G*, probes for tRNA Lys, Glu, and Gly, respectively. *scR1*, the three membranes were reprobed with scR1 for normalization. The origin of each RNA sample is shown at the *top*; the *five lanes* on the *left* are from strains constructed in the W303 background, and the *four lanes* on the *right*, with *asterisks*, are in the S288c background. *wt*, wild type. *B*, a diploid strain heterozygous for the *trm112-0* deletion was transformed with high copy number plasmids expressing $tRNA^{Lys}$, $tRNA^{Glu}$, $tRNA^{Gln}$, or the three tRNA together (*EKQ*), as indicated. These strains were then sporulated, the tetrads were dissected, and the growth of the resulting spores containing the plasmids was tested at 30 °C. Shown here are the results for two tetrads (*lanes a* and *b*) for each transformant. The large colonies (such as *lane a*, *rows 1* and *4*, and *lane b*, *rows 1* and *3*, for $tRNA^{Lys}$, for instance) were tested and found to be wild type for *TRM112*, whereas the small ones were *trm112*::*kanMX4*. None of the tRNA expressing plasmids was able to compensate for the growth defect of the *trm112-0* strain.

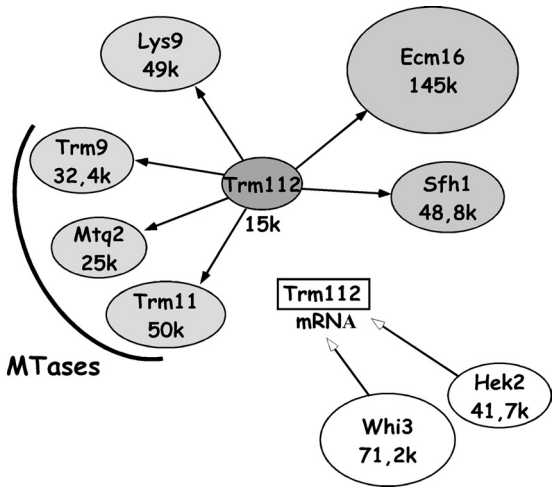

FIGURE 6. **Partners of Trm112p.** The figure schematically represents six putative partners of Trm112p. The four proteins represented in *light shading* correspond to the ones that were predicted to be Trm112p partners when we initiated this study (10, 34) and that were then confirmed individually by co-immunoprecipitation. They all possess a Rossman fold domain that is the signature of enzymes utilizing *S*-adenosylmethionine as a co-factor, and they are all confirmed MTases, except for Lys9p, which lacks one of the seven β-strands of the fold. The proteins shown in *darker shading* were reported more recently and do not possess a Rossman fold. Ecm16/Dhr1p was shown to interact with Trm112p in a recent proteomic analysis (52). It is a DEAH-box ATP-dependent RNA helicase involved in rRNA maturation (53). Sfh1p was then reported to interact with Trm112p in a two-hybrid interactome network (36). It is a component of the RSC complex that is phosphorylated during the G_1 phase of the cell cycle. It is encoded by an essential gene, and the protein is required for cell cycle progression and maintenance of proper ploidy (37, 38). Two more proteins are represented on a white background, with *arrows* pointing to TRM112 mRNA. Hek2/Khd1p and Whi3p are RNA-binding proteins that were found to participate in the localization of numerous mRNA at the tip of the bud (54) and at the endoplasmic reticulum (55), respectively.

Therefore, we decided to investigate whether the steady-state level of certain tRNA is altered in the strains mutated for *TRM9*, *MTQ2*, or *TRM112*, thus explaining the growth defect of these mutants. Quantitative Northern blot analyses were performed using probes for 15 different tRNAs: class I tRNAs possess a thiolated modification of the wobble base (mcm^5s^2U: $tRNA^{Gln}_{UUG}$, $tRNA^{Glu}_{UUC}$, and $tRNA^{Lys}_{UUU}$), class II tRNAs possess a mcm^5U at that position ($tRNA^{Arg}_{UCU}$ and $tRNA^{Gly}_{UUC}$), and class III tRNAs do not contain a modified U at the wobble position ($tRNA^{Ser}_{CGA}$, $tRNA^{Met}_{Aug}$, $tRNA^{His}_{GUG}$, $tRNA^{Ala}_{IGC}$, $tRNA^{Trp}_{CCA}$, $tRNA^{Phe}_{GAA}$, $tRNA^{Asn}_{GUU}$, $tRNA^{Ile}_{IAU}$, $tRNA^{Leu}_{GAA}$, and $tRNA^{Leu}_{CAA}$). Northern blots were quantified by phosphorimaging and standardized using a probe for scR1, the 522-nucleotide-long RNA component of the signal recognition particle that is a very stable RNA, relatively insensitive to physiological variations (35). Although for nucleoside modification analyses tRNA was prepared by treating the whole cells with phenol, a procedure that renders the cell permeable to tRNA and leads to considerable enrichment (28), here we prepared total RNA to ensure that no quantitative variation could be attributed to the extraction procedure. Fig. 5*A* represents the result of a typical experiment in which tRNA was prepared from two series of mutant strains; the *five lanes* on the *left* correspond to the W303 background, and the *four lanes* on the *right* correspond to the S288c background. Shown here are the results for two tRNA that contain the thiolated modification mcm^5s^2U ($tRNA^{Lys}_{UUU}$ and $tRNA^{Glu}_{UUC}$) and one containing the nonthiolated mcm^5U ($tRNA^{Gly}_{UUC}$). There are only small variations in tRNA levels between the different mutant strains and no major differences between the W303 and the S288c backgrounds (supplemental Fig. S3). Then, because it was shown that certain mutants affecting the formation of mcm^5s^2U could be rescued by overexpressing the tRNA containing these modifications, we expressed high levels of $tRNA^{Gln}_{UUG}$, $tRNA^{Glu}_{UUC}$, $tRNA^{Lys}_{UUU}$, or a combination of the three either in a *trm112-0* strain or in a strain with the double deletion *mtq2-0 trm9-0*. The results clearly indicate that tRNA overexpression was not sufficient to rescue the normal growth of the mutant cells (Fig. 5*B*).

Nuclear Genomic Instability and Mitotic Defects in the trm112-0 Mutant—In addition to its role as an MTase co-factor, Trm112p has also recently been found in a two-hybrid screening as an interactor of Sfh1p (Fig. 6) (36), an essential component of the "remodels the structure of chromatin" (RSC) complex (37), which is involved in transcription and is also necessary for proper mitosis (38, 39). Because no functional data were available for the potentially relevant Trm112p-Sfh1p interaction, we examined *trm112-0* mutant phenotype in comparison with a conditional mutant allele (degron) of the *SFH1* gene (40). Full inactivation of that degron allele of *SFH1* leads to both G_1 and G_2/M cell cycle arrest. Therefore, because a *trm112-0* mutant grows slowly but is viable, we compared it with an $sfh1^{td}$ mutant grown under semi-restrictive conditions ($sfh1^{td}$ P_{GAL}::*UBR1* on galactose at 30 °C). Nuclear staining of

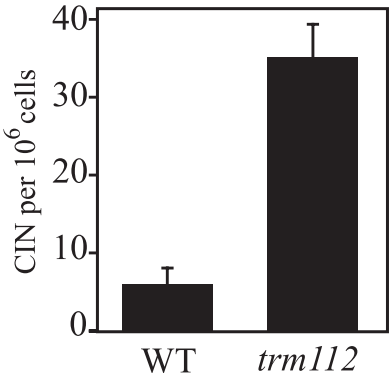

FIGURE 7. **Nuclear genome instability in WT *versus trm112-0* mutant.** The values represent the rates of CAN 5-fluoro-orotic acid-resistant clones/million cells (see "Experimental Procedures" and Ref. 27), indicative of chromosome break or loss of the distal arm on chromosome V. *CIN*, chromosome instability; *WT*, wild type.

log phase cultures showed a class of cells present in both *sfh1*^{td} and *trm112-0* mutants but absent in the isogenic wild-type control (supplemental Fig. S4). Those cells are characterized by a split nucleus within the mother cell, which indicates that anaphase takes place in the absence of proper nuclear positioning relative to the mother/daughter axis (41). This is most likely due to a defective regulation of either cytosolic microtubules or cortical proteins. The percentage of cells affected is seemingly low (~1–2% in both strains) but very significant compared with the frequency in wild-type cells (below 0.1%). Such misoriented anaphase events had also been observed using *sfh1*^{td} as well as other mutants of the RSC complex (39). This observation, although preliminary, suggests that the Trm112p-Sfh1p interaction might be functionally relevant.

Using a reporter strain designed for chromosome instability measurements,[4] similar to the assay described previously (27), we then quantified chromosome instability events in the absence of *TRM112* (Fig. 7). This assay measures the loss of a chromosome arm containing two counter-selectable markers. Chromosome instability increases 6–7-fold in *trm112-0* compared with wild type, similar to the chromosome instability measured in a RSC mutant using a different assay (39). This is consistent with a potential role for Trm112p as a positive regulator of the RSC complex activity.

DISCUSSION

In recent years, proteomics analyses have proven to be powerful tools to reveal the existence of networks connecting proteins that were previously not known to operate in the same pathways. Such a study, by revealing the existence of four partners for Trm112p, has opened the road to the discovery that Trm112p is required for the activity of Trm11p, Mtq2p and, as shown here, Trm9p. When Kalhor and Clarke (9) identified Trm9p as being responsible for the last step of mcm^{5}U and mcm^{5}s^{2}U formation, they also proposed that the enzyme was sufficient to catalyze this reaction because they were able to detect some activity *in vitro*, using a recombinant protein prepared from *E. coli*. We have re-examined this statement based on the data presented in this report. First, our data show that mcm^{5}U and mcm^{5}s^{2}U are undetectable in a *trm112-0* strain, similar to a *trm9-0* strain. This result corroborates the observation that a *trm112-0* strain is resistant to zymocin (13), the tRNase toxin produced by *K. lactis* that specifically targets the mcm^{5}s^{2}U modification at position 34 and cleaves tRNA$^{Gln}_{UUG}$, tRNA$^{Glu}_{UUC}$, and tRNA$^{Lys}_{UUU}$. Second, the level of activity reported for the recombinant Trm9p alone was extremely low (9). Even this low level of activity may not be entirely attributable to Trm9p alone because we previously indicated that there is a protein in bacteria that might be evolutionarily related to Trm112p and thus be an accessory factor for Trm9p, although much less efficiently than Trm112p (2). Third, although Trm9p expressed alone in *E. coli* is mostly insoluble, we report here that co-expressing the two recombinant proteins Trm9p and Trm112p leads to the formation of a soluble complex that possesses a very strong catalytic activity *in vitro*. Recently, it was reported that Trm11p alone, when produced in a wheat germ extract, had no tRNA methylation activity, whereas this activity was detected when Trm11p was synthesized together with Trm112p (8). Trm112p was also shown to be required for the activity of Mtq2p, the MTase of eRF1 (7). Therefore, Trm112p is essential for the activity of at least three different enzymes.

Trm112p contains a zinc finger domain, a motif composed of four cysteines arranged with a certain spacing, which can form a secondary structure resembling a finger. Phylogenetic comparison has led to the assumption that this structure is not conserved in higher eukaryotes. However, crystal structure solution of yeast Trm112p confirmed the presence of a zinc atom coordinating the four cysteines Cys-11, Cys-16, Cys-112, and Cys-115, as previously hypothesized (2, 7). The zinc finger domain was first identified 25 years ago in the transcription factor TFIIIA from *Xenopus laevis* that is required for the transcription of the 5 S rDNA genes by RNA polymerase III (42). TFIIIA contains nine zinc fingers, some of which are devoted to DNA binding and some of which, interestingly, are devoted to RNA binding (43). Numerous zinc finger-containing proteins have now been reported, most of which are transcription factors, such as Gal4p in yeast (44, 45). Several zinc finger proteins are RNA-binding proteins that are involved in a variety of processes including viral proteins, mRNA metabolism, etc. (46). Our data clearly show that Trm112p is required for the activity of two tRNA MTases, Trm11p and Trm9p. It is thus tempting to hypothesize that Trm112p, because of the presence of a zinc finger, could be responsible for tRNA binding, bridging the tRNA template and the modification enzyme. However, it should be noted that the two modified nucleosides, at positions 10 and 34, are located in very different contexts in the tRNA. Position 34 is in the anticodon loop that is well exposed and very accessible in the tRNA L-shaped structure, whereas position 10 is in a groove, at the junction between the acceptor loop and the D-loop. Therefore, Trm9p/Trm112p and Trm11p/Trm112p not only recognize different nucleotides (cmU and G) surrounded by very different sequences on the tRNA but also interact with very different secondary/tertiary structures, making it unlikely that Trm112p is dedicated to target recognition. Several other points should also be considered. Trm112p seems to be essential for these enzymes to fold properly, as shown by their co-expression in *E. coli*, an observation suggesting that Trm112p is an intrinsic component of these enzymatic activities rather than an accessory factor bonding these proteins to

their specific targets by providing them sequence recognition. Also, Trm112p is essential for Mtq2p folding and activity, although Mtq2p is a protein MTase, not a tRNA one, a result that weakens the idea that Trm112p could be solely involved in nucleic acid recognition. Interestingly, the protein target for Mtq2p is eRF1/Sup45, a protein that adopts a tRNA-like shape (47) and can enter the ribosome at the A-site. However, if Trm112p was responsible for tRNA recognition of the two MTases Trm9p/Trm112p and Trm11p/Trm112p, it would be unlikely that it plays the same role in binding eRF1/Sup45; adopting a tRNA-like shape or being a true tRNA are likely to be very different at the molecular level. In addition, the recent discovery that Sfh1p could be another partner of Trm112p does not point, at the moment, to an RNA binding activity for Trm112p (36). It is not yet clear what makes these three enzymes insoluble when they are produced alone. Further structural characterization of these enzymes complexed to Trm112p will be necessary to determine which domain interacts with Trm112p and thus propose a model for the action of this small protein.

Trm11p and Trm1p catalyze the formation of m^2G_{10} and $m^2{}_2G_{26}$, respectively, two modifications that are found simultaneously in a number of tRNAs ($\geq$14) and can possibly interact with each other when the tRNA has acquired its proper L-shaped structure (2). Supporting these observations, we previously described a genetic link between the two genes encoding these enzymes because *trm11-0* exhibits a synthetic growth defect when combined with *trm1-0* (2). In contrast, it is quite puzzling that Trm11p and Trm9p share a subunit because nothing else appears to obviously link these two activities. Their targets are very different in nature and are located in different contexts, and they do not affect the same tRNAs, because of five tRNAs having either mcm^5U_{34} or $mcm^5s^2U_{34}$, only two also have an m^2G_{10}. Finally, *trm11-0* and *trm9-0* do not exhibit any synthetic growth defect.

More surprisingly, *trm9-0* exhibits a synthetic growth defect with *mtq2-0*, thus establishing an unexpected genetic link between tRNA biogenesis and termination of translation, a conclusion that is also supported by the fact that they share a subunit, Trm112p. It has been proposed recently that the availability of Trm112p is limiting in the cell and that the level of its different partners is important to maintain the balance between their activities. For instance, overproduction of Mtq2p would affect the activity of Trm9p by depriving it of Trm112p (13). However, this would not explain the synthetic effect we observed between *mtq2-0* and *trm9-0*, because in that case, Trm112p should not be in a limiting amount.

tRNA modifications have been implicated in various aspects of tRNA biogenesis including controlling the steady-state levels of certain tRNAs, which in turn might considerably affect the expression of a population of mRNA containing the corresponding complementary codons. For instance, the formation of m^1A_{58} that is catalyzed by the complex Trm6p/Trm61p, which is detected in approximately two-thirds of the tRNAs in yeast, appears to be important only for the stability of the initiator tRNA methionine ($tRNA_{ini}^{Met}$). Moreover, overexpression of $tRNA_{ini}^{Met}$ is able to bypass the deletion of either *TRM6* or *TRM61*, which otherwise are essential genes (5). Also it is possible to suppress the activity of the killer toxin, which otherwise cleaves the tRNA containing mcm^5s^2U at the wobble position, by overexpressing the corresponding tRNAs (19, 21). Therefore we investigated whether there was a significant variation of the steady-state levels of several tRNAs that could explain the reduced growth rates of either the double mutant *mtq2-0 trm9-0* or the single mutant *trm112-0*. Also, we overexpressed three tRNA containing the mcm^5s^2U modification in these mutants to test whether they were sufficient to rescue their growth. Our results show that there is no major variation in tRNA abundance and that overexpression of the target tRNA did not rescue the growth of the *trm112-0* or the *mtq2-0 trm9-0* mutants.

The synthesis of the mcm^5 group is complex and requires the activity of many genes (14). It has been reported that recombinant yeast Trm9p expressed in *E. coli* catalyzes the formation of both mcm^5U and mcm^5s^2U when using saponified tRNA as a substrate (9). Such a substrate is likely to contain cm^5U and cm^5s^2U. From these experiments it was not possible to determine how efficient the reaction was because the level of the possible intermediates in the saponified tRNA was not determined. Moreover, mild base treatment of a tRNA may also induce other changes in its structure. The methylation of the saponified tRNA was poor, but still both mcm^5U and mcm^5s^2U were made, although no Trm112p was present. We used as a substrate tRNA from the *trm9-0* mutant and observed an efficient and quantitative synthesis of mcm^5U using the recombinant Trm9p/Trm112p heterodimer as enzyme. However, no mcm^5s^2U was formed, contrary to the results obtained using saponified tRNA as substrate. Because our substrate did not contain cm^5s^2U, which is likely to be the immediate precursor to mcm^5s^2U, the inability to form mcm^5s^2U is understandable. The discrepancy between our result and the results obtained with saponified tRNA may therefore be due to the different substrates and to the fact that we used a Trm9p/Trm112p heterodimer instead of only Trm9p as in the earlier study (9). The presence of Trm112p may induce a specificity of the methylation reaction, *e.g.* sensitivity to the s^2 group, which is not present in the Trm9p peptide. Interestingly, similar results were recently obtained using a human Trm9p/Trm112p heterodimer and tRNA from a human *trm9-0* strain ($ALKBH8^{-/-}$) as substrate (48).

The amide derivatives (ncm^5U and ncm^5s^2U) of the potential immediate substrates (cm^5U and cm^5s^2U) for the Trm9p/Trm112p heterodimer accumulated in tRNA from *trm9-0* and *trm112-0* mutants (Table 1) consistent with earlier reports (11, 48). *In vitro* these compounds are not substrates for the Trm9p/Trm112p heterodimer because their levels in tRNA did not decrease upon enzyme treatment (Table 2). The accumulation of these compounds in the *trm9-0* and *trm112-0* mutants may be due to a slow aberrant amidation of cm^5U and cm^5s^2U generated by the Trm9p or Trm112p deficiency. Alternatively, these compounds may be generated prior to the synthesis of cm^5U and cm^5s^2U in the biosynthetic pathway to form the mcm^5 side chain. Clearly, more studies must be performed before we understand the complex synthesis of the mcm^5 side chain that involves so many gene product activities.

Refined genome-wide interactome of *S. cerevisiae* points to Sfh1p as a potential Trm112p partner (36). *SFH1* is an essential gene, whose product is part of the RSC complex (38, 39). If it acts as a positive regulator of RSC, Trm112p inactivation might therefore recapitulate some of the phenotypes observed in RSC mutants, especially in *sfh1*. Our data indeed show that unusual mitotic figures, observed in RSC mutants, are also present in *trm112-0* (supplemental Fig. S4). Nuclear genome instability, a common hallmark of cell cycle perturbation, is increased both in *trm112-0* (Fig. 7) and in RSC mutants (39). Our preliminary observations are consistent with a functional link between Trm112p and Sfh1/RSC. The occurrence of rare "endomitosis" in both mutants suggests that Trm112p could be involved in regulating the position of the nucleus relative to the normal division axis. RSC is also involved in multiple processes including transcription (49), kinetochore function (39), and cohesion between sister chromatids (50). It will be of interest to see whether Trm112p is involved in any of these functions. Recently, SMO2, a *TRM112* homolog, has been identified in *Arabidopsis*; smo2 knock-out reduces cell division without affecting differentiation (51). Elevated levels of B type cyclins suggest a defect of smo2 cells during mitosis. This supports a mitotic role for Trm112p, potentially conserved throughout evolution.

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