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Research paper

RNA polymerase III transcription machinery and tRNA processing are regulated by the ubiquitin ligase Rsp5

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ABSTRACT

Transfer RNA (tRNA) biogenesis in yeast involves synthesis of the primary transcript by RNA polymerase III (Pol III), followed by processing to remove 5′ and 3′ ends, further maturation, and export to the cytoplasm. In the present study, we found that both tRNA transcription and the initial processing of tRNA precursors are affected by the ubiquitin ligase Rsp5. We observed high levels of unprocessed primary tRNA transcripts in *rsp5* mutants at elevated temperature, which were reduced upon the overexpression of *RPR1*, the catalytic subunit of RNase P. This observation suggests a role for Rsp5 in the maturation of 5′ ends of tRNA precursors. Under the same conditions, in vivo labeling showed that the amount of newly synthesized tRNA decreased. Furthermore, we found that Rsp5 directly interacted with the Tfc3 subunit of the TFIIIC transcription factor, which is modified by ubiquitination. The inactivation of Rsp5 catalytic activity influenced the interaction between the general Pol III factors TFIIIB and TFIIIC and decreased the recruitment of TFIIIC to tRNA genes. These findings suggest that Rsp5 ligase is implicated in the control of Pol III transcription in yeast.

1. Introduction

RNA polymerase III (Pol III) is the largest of eukaryotic RNA polymerases and responsible for the transcription of small non-coding RNAs, mainly transfer RNAs (tRNAs). The Pol III-mediated transcription of tRNA genes is under the control of TFIIIB and TFIIIC transcription factors and the negative regulator Maf1 protein (for review, see [1]). The initiation of Pol III transcription begins with the binding of TFIIIC, a large protein complex that is composed of six subunits that form two subcomplexes, tau A and tau B, to intragenic tRNA promoter elements, called A- and B-boxes [2]. Once TFIIIC anchors to the tRNA gene, the flexible linker region between the tau A and tau B subcomplexes allows TFIIIC binding to the A-box which is variably positioned upstream of the A-box [3]. The initiation factor TFIIIB, which consists of three subunits, then forms a stable complex only when all subunits are bound to DNA [4]. Next, the contact between the N-terminal region of Tfc4, a subunit of A-box and TFIIIB, is broken, and TFIIIC tau A dissociates from the DNA. At the same time, the stable TFIIIB-DNA complex is assembled and can recruit Pol III [5].

Pol III machinery is tightly regulated by the nutrient and energy status of cells, and several signaling pathways control Pol III activity. One of the major targets of these pathways is the master regulator of Pol III transcription, Maf1, which represses the synthesis of tRNAs under a wide range of cellular and environmental stress conditions [7–10]. The dysregulation of tRNA synthesis in mammals leads to numerous pathological states, including cancer and Pol III-related leukodystrophy [6].

Numerous components of yeast Pol III machinery are subjected to post-translational modifications, such as phosphorylation, sumoylation, ubiquitination, and methylation, which impact transcriptional activity and might correlate with changes in environmental conditions (for review, see [11,12]). Knowledge of the ways in which Pol III machinery is regulated by ubiquitination is very limited. The largest Pol III subunit, C160, is ubiquitinated and degraded by the proteasome under conditions that are associated with Pol III repression [13,14]. Tbp1, a TATA-binding protein and component of TFIIIB, undergoes ubiquitination and degradation [15], but unclear is whether the ubiquitination of Tbp1 affects tRNA transcription.

Several ubiquitin-protein ligases have been identified in the control of Pol II transcription in yeast [16,17], but their role in Pol III regulation has not been explored. Previous findings show that Rsp5, a HECT domain-containing E3 ubiquitin ligase, is a multifunctional enzyme that is implicated in the regulation of transcription and many cellular processes (for review, see [18–20]). Rsp5 is a cytoplasmic-nuclear shuttling protein [21] that is involved in tRNA metabolism [22]. The

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accumulation of tRNA primary transcripts was observed in *rsp5* mutants under restrictive conditions [22,23], suggesting a role for Rsp5 in tRNA maturation and export.

Several links between Rsp5 ligase and tRNA biogenesis have been identified by large-scale proteomics (for review, see [20]). Interestingly, several subunits of Pol III and its general factors, TFIIIB and TFIIIC, are potential substrates for Rsp5. Furthermore, other candidates for ubiquitination by Rsp5 ligase include proteins that are involved in tRNA maturation, including those that engage in tRNA 5′- or 3′-end processing and tRNA transport [24–26].

The present study sought to provide insights into the role of the ubiquitin ligase Rsp5 in the function of Pol III transcription machinery and tRNA biogenesis. We explored the effect of *rsp5* mutants on the accumulation of tRNA primary transcripts to distinguish between tRNA transcription and maturation, hypothesizing that both processes are affected. Next, we found a direct link between Rsp5 ligase and Pol III machinery through interactions with Tfc3, a subunit of the TFIIIC transcription factor. We also investigated the effects of Rsp5 on the Tfc3 occupancy of tRNA genes and interactions between TFIIIB and TFIIIC transcription factors to elucidate a possible regulatory mechanism. Detailed analyses of precursors of different tRNA species that accumulated in the *rsp5* mutant suggested the influence of Rsp5 on the early step of tRNA maturation that involves RNase P.

2. Materials and methods

2.1. Yeast strains, plasmids, media and growth conditions

MHY501, wild-type strain (*MAT*α *his3*Δ*200 leu2-3,112 ura3-52 lys2-801 trp1-1*) and isogenic *rsp5-1* [27] and *rsp5-19* [28] mutants were kindly supplied by Teresa Żołądek (IBB, PAS). *maf1*Δ mutant with inactivation *MAF1* gene was described previously [7]. SC0000, wild-type W303 derived parental strain for TAP fusion strain and Tfc3-TAP (*MATa ade2,* arg*4, leu2-3,112, trp1-289, ura3-52, YAL001c::TAP-KlURA3*) were purchased from the Euroscarf collection. MC80-2A and MC81-1A strains containing Tfc3-TAP *rsp5-1* and Tfc3-TAP *rsp5-19* were constructed by the cross of Tfc3-TAP with *rsp5-1* and *rsp5-19*, respectively. MC102-1D strain carrying Tfc4-GFP Bdp1-HA *rsp5-1*, was constructed by the cross of MC61bis-6A (*MAT*a *leu2 ura3 met15 ade2-1 TFC4-GFP*::*HIS3 BDP1-3HA*::*KanMX6*) [29] with *rsp5-1* mutant. MC39-3B strain (*MAT*a *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 tfc3*::*KanMX4* [*TFC3-TAP LEU2/CEN*]) [29] was transformed with plasmids bearing the *RSP5* or *rsp5-19* mutant version and the triple hemagglutinin (HA)-epitope tag, *RSP5* [30] and *HA-rsp5-19* [31], respectively. MK1 strain produces a genomic version of Rsp5 with an N-terminal HA fusion [31]. For tagging C160 with HA in wild-type, MHY501 and isogenic *rsp5-1* strain, the appropriate sequence was amplified by PCR, using the genomic DNA of MW4415 strain encoding C160-HA (*MAT*a *ura3-52 his3-Δ200 ade2-101 trp1-Δ63 lys2-801 leu2-Δ1 C160*::*3HA*::*KanMX6*) as template and specific primers, C160F (5'-GGACGCTGTTGAAGGTGTTT-3') and C160R (5'-AAACGTTGACGCGAGTTTTT-3'). Transformants were selected on YPD medium supplemented with geneticin (200 μg/ml). The HA sequence was confirmed by PCR and by Western blot method with HA antibodies (Convance).

Overexpression of a permease gene, *FUI1* was performed from a pRS425-*FUI1* (ampR 2 μ *LEU2*) plasmid [32], overexpression of *RPR1* gene from a pFL44L-*RPR1* (ampR 2 μ *URA3*) plasmid [33] and overexpression of His-tagged ubiquitin from a YEp96-6His-Ub (ampR 2 μ *TRP1 CUP1*) plasmid [34].

The following yeast media were used: YPD (2 % glucose, 2 % peptone, 1 % yeast extract), YPGly (2 % glycerol, 2 % peptone, 1 % yeast extract), SC-trp and SC-ura minimal media (2 % glucose, 0.67 % yeast nitrogen base, supplemented with 20 μg/ml of all the amino acids required for growth, or all except for tryptophan or uracil, respectively), sporulation medium (0.05 % glucose, 0.1 % yeast extract, 1 % potassium acetate). Solid media contained 2 % agar. All reagents used for media were Difco products.

The growth rate of yeast strains in liquid cultures was monitored by optical density measurements OD_{600}. Cells were grown at 30 °C to the exponential growth phase (OD_{600} ~ 0.5–0.7) and then transferred to 37 °C for 4 h or transferred to YPGly medium at 37 °C for 2 h. The *CUP1* promoter was induced with 100 μM copper sulfate. In the cycloheximide (CHX) chase assay, cells were treated with 100 μg/ml CHX.

2.2. Northern blotting

RNA isolation and Northern hybridization were done as described previously [35]. Quantity and purity of the RNA were analyzed using NanoDrop 1000 (Thermo). A 5 μg aliquot of the total RNA was separated by electrophoresis on 10 % polyacrylamide, 8 M urea gel. RNA was transferred from the gel onto a Hybond-N+ membrane (Amersham) by electroblotting in 0.5 × TBE and crosslinked by UV radiation. The following DIG-labeled oligonucleotides were used for RNA hybridization: tRNA Lys(UUU) 5'-ATCCTTGCTTAAGCAAATGCGCT-3', tRNA Leu (CAA) 5'-TATTCCCACAGTTAACTGCGGTCA-3', tRNA Tyr(GUA) 5'-GAGAGTCGATTTCTTGC-3', tRNA Trp(CCA) 5'-GCAATCTTATTCCGTG-GAATTTCCAAG-3', *RPR1*, 5'-GGCTCCACTGTGTTCCACCGAATTTCC-CAC-3' and 5.8S rRNA, 5'-GCGTTGTTCATCGATGC-3'. The primer sequences are complementary to the introns of tRNA Lys(UUU), tRNA Leu(CAA), tRNA Trp (CCA) and therefore precursor forms of tRNAs were detected by Northern blot analysis. Only tRNA Tyr(GUA) probe detects precursors and mature form of tRNA.

2.3. Labeling and purifying newly synthesized RNA

Isolation of newly synthesized RNA using 4-thiouracil was based on [32,36]. Wild-type and *rsp5* mutants cells transformed with pRS425-*FUI1* plasmid were grown in SC-ura medium overnight at 30 °C and then transferred to YPD medium, grown at 30 °C to the exponential phase and shifted to 37 °C for 4 h. 4tU (Sigma) was added to a final concentration of 100 μM for 10 min. Next cells were cooled by pouring cultures into 75 ml of cold 96 % ethanol. RNA isolation was done as described previously [35]. All samples were prepared in three repetitions and additionally, a sample without 4tU labeling was analyzed to check the background RNA amounts. *S. pombe* RNA spike-in (total RNA from cells grown to the exponential phase in YPD medium at 30 °C and labeled with 4tU for 10 min) was added to all samples for normalization, 1 μg of *S. pombe* RNA per 100 μg of *S. cerevisiae* RNAs. Around 150–200 μg of RNA was resuspended in 400 μl 1 × TE buffer. Biotinylation, desalting and IP sample preparation were performed as described previously [36]. For IP 50 μl of Dynabeads MyOne Streptavidin C1 (Invitrogen) was used per sample. After IP reaction RNA was eluted with 50 μl of 0.7 M β-mercaptoethanol for 5 min and precipitated with 280 μl 96 % ethanol, 10 μl 3 M sodium acetate pH 5.3 and 2.5 μl 20 mg/ml glycogen. Next RNA was resuspended in DEPC-treated TE buffer with SUPERase-In (Invitrogen). cDNA was synthesized by reverse transcription and analyzed by RT-qPCR as described in [37]. Values were normalized to input RNA concentrations and to the levels of SpACT1 mRNA encoding actin of *S. pombe*, which was used as an internal control (oligonucleotide sequence: Act1_S.pombe_fwd 5'-CCATTCTTGCTTCTCTTTCTACTTTCC-3′, Act1_S.pombe_rev 5'-CGCTCTCATCATACTCTTGCT-3′). The following oligonucleotides were used to analysis of tRNA synthesis: tLeu (*CAA*)G1_For 5'-AGAACCGAAACATACAAATAAGTGGT-3′, tLeu(*CAA*) G1_Rev 5'-TGATCACAGAACCAAAAAGATAAAA-3′, tPhe(*GAA*)H1_For 5'-TCAGAATTTCAACAAATAGTAAGCGGA-3′, tPhe(*GAA*)H1_Rev 5'-TGCCCTGTCATGTTAGGAAATACA-3′, tHis(*GUG*)E1_For 5'-CCTTTATCGTCTAGTTACACCAGTAGTC-3′, tHis(*GUG*)E1_Rev 5'-CCATGTACAAACCACCGATAAG-3′.

2.4. Co-immunoprecipitation Tfc3 and Rsp5

Two different immunoprecipitation (IP) assays were used: 1) total

extracts isolated from wild-type, *rsp5* mutants encoded TAP-tagged Tfc3 were incubated with magnetic beads coated with anti-Tfc3 antibody. The extract isolated from the untagged wild-type strain was IP using magnetic beads coated with pre-immunoserum and served as a negative control. Additionally, total extracts isolated from an untagged wild-type strain and from a strain producing Rsp5 with an HA fusion were IP using anti-Tfc3 antibody-coated beads. Co-purified proteins were detected using anti-Tfc3, anti-TAP, anti-Rsp5 and anti-HA antibodies. 2) Total extracts isolated from *tfc3*Δ [*TFC3-TAP*] cells transformed with plasmids encoding *HA-RSP5* and *HA-rsp5-19* were IP using magnetic beads coated with anti-HA antibody and co-purified proteins were detected using anti-HA and anti-TAP antibody. Yeast cells were grown in the YPD or SC-trp to the exponential phase at 30 °C, for 1) and 2) experiment, respectively. Pellets harvested from 50 ml cultures were resuspended in 0.5 ml of IP buffer (50 mM HEPES-KOH pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.05 % NP-40, 0.5 mM DTT, 5 % glycerol, O-Complete protease inhibitor [Roche]). Isolation of total extracts and measurement of protein concentration were performed as described in [38]. 50 μl of Dynabeads anti-Rabbit IgG magnetic beads or Dynabeads anti-Mouse IgG magnetic beads (Invitrogen) for 1) and 2) experiment, respectively, were washed three times with 0.5 % bovine serum albumin in phosphate-buffered saline (PBS) and incubated for 3.5 h at 4 °C with 3 μg rabbit anti-Tfc3 antibody and 3 μg rabbit anti-pre-immune serum or 3 μg mouse anti-HA antibody for 1) and 2) experiment, respectively. After washing in 0.5 % bovine serum albumin in PBS (two times) and then in IP buffer (two times), the beads were incubated overnight at 4 °C with 2 mg of protein extracts in IP buffer with gentle shaking and then washed three times with IP buffer. The IP proteins were released from the beads by boiling them for 5 min and separated by 6 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting.

2.5. Identification of ubiquitinated Tfc3 species using Ni-NTA agarose and denaturing conditions

Wild-type cells producing Tfc3-TAP, optionally transformed with [His-Ub] plasmid under *CUP1* promoter were grown in SC-trp medium at 30 °C. Expression of tagged ubiquitin was induced by copper sulfate to a final concentration of 100 μM, then cells were grown for 3 h to the exponential phase. 300 ml of cultures were harvested, washed with PBS buffer and stored at −80 °C for further analysis. Pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 7.8, 300 mM NaCl, 0.1 % NP-40, 5 mM $MgCl_2$, 6 M guanidine hydrochloride, 10 mM imidazole, 0.2 mM MG132, 5 mM β-mercaptoethanol) with O-Complete protease inhibitor (Roche) at the rate of 1 ml per 100 optical density of yeast cells. Isolation of total extracts, measurement of protein concentration and the buffers used in subsequent IP steps, i.e. WBI, WBII and EB, were performed as described previously [13]. 2 mg of protein extract was added to the 200 μl Ni-NTA agarose (Qiagen), washed two times with 1 ml WBI buffer and incubated o/n at 4 °C with rotation. Washing of Ni-NTA agarose and elution of proteins were performed using poly-prep columns (Bio-Rad). Agarose was transferred into the columns and washed three times with 1 ml WBI buffer and two times with 1 ml WBII buffer. Then, proteins were eluted twice with 400 μl EB buffer and precipitated with TCA, then resuspended in 30 μl 2 × SB buffer containing 6 M urea and boiled for 5 min at 96 °C. Proteins were separated by 6 % SDS-PAGE and analyzed by Western blotting with anti-TAP and anti-Ubi antibodies.

2.6. Affinity of ubiquitinated forms of Tfc3 by using magnetic beads and non-denaturing conditions

Wild-type cells producing Tfc3-TAP and transformed with the [His-Ub] plasmid were grown at 30 °C to the exponential phase, supplemented with 100 μM copper sulfate and then shifted to 37 °C for 4 h. Tfc3 ubiquitinated protein was purified using Dynabeads anti-rabbit IgG magnetic beads (Invitrogen) coated with a Tfc3-specific antibody as described in Material and Methods: Co-immunoprecipitation Tfc3 and Rsp5. Ubiquitinated forms of Tfc3-TAP were detected by Western blot with anti-TAP antibody.

2.7. Immunoprecipitation of Tfc4-GFP

To faithfully establish the assembly-recruitment relationship between TFIIIC and TFIIIB, the interaction in cross-linked chromatin was analyzed as previously described [9,29]. Wild-type and *rsp5-1* cells producing GFP epitope-tagged Tfc4 and HA epitope-tagged Bdp1 and untagged wild-type strain were cross-linked with 1 % formaldehyde for 30 min at room temperature, washed twice in PBS and pelleted. Isolation of protein extracts, preparation of magnetic beads, IP (anti-GFP) and elution from beads were done as described previously [29]. Total protein extracts and IP proteins were separated by 8 % SDS-PAGE and hybridized with mouse anti-GFP, anti-HA, anti-Pgk1 and with rabbit antibody against Brf1.

2.8. Western blotting

Protein extracts and IP fractions were separated on SDS-PAGE using acrylamide:bisacrylamide ratio of 37.5:1. After electrophoresis proteins were transferred onto the nitrocellulose membrane (Millipore), which was then blocked in TBST (10 mM Tris, 150 mM NaCl, 0.05 % Tween 20) containing 5 % fat-free dry milk for 30 min and subsequently incubated with the appropriate antibody: mouse antibodies anti-HA (Covance, cat. no. 901503) at a 1:5000 dilution for o/n at 4 °C, anti-GFP (Roche, cat. no. 11093274910) at a 1:5000 dilution for o/n at 4 °C, anti-Vma2 (Invitrogen, cat. no. A6427) at a 1:5000 dilution for 1 h, anti-Pgk1 (Invitrogen, cat. no. 459250) at a 1:10000 dilution for 1 h, rabbit antibodies anti-Tfc3 (Cusabio, made to order) at a 1:2000 dilution for o/n at 4 °C, anti-Ubi (Sigma, cat. no. U-5379) at a 1:2000 dilution for o/n at 4 °C, anti-Brf1 (Gramsh, made to order) at a 1:1000 dilution for o/n at 4 °C. Blots with TAP-tagged Tfc3 were incubated with peroxidase anti-peroxidase (PAP) rabbit antibody (Sigma-Aldrich, cat. no. P129-1ml) at a 1:5000 dilution for 1 h. Rsp5 protein was detected using rabbit antibody against Nedd4 WW2 (Millipore, cat. no. 07–049) at a 1:5000 dilution for o/n at 4 °C. Then, nitrocellulose membranes were incubated with secondary anti-mouse (Dako, cat. no. P0447), or anti-rabbit (Dako, cat. no. P0448) antibodies coupled to horseradish peroxidase at a dilution of 1:5000 for 1 h and visualized by chemiluminescence using the ECL detection kit (Bio-Rad).

2.9. Chromatin immunoprecipitation and quantitative PCR

Chromatin isolation and immunoprecipitation from the yeast cells producing TAP epitope-tagged Tfc3 and HA epitope-tagged C160 were performed as described previously [29]. The input and immunoprecipitated samples were assayed by quantitative PCR to assess the extent of Tfc3 and C160 protein occupancy at different genomic regions. The sequences of the primers used in this study were: tMet(*CAU*)E_For 5'-GCGGACCTCAATATAAGCGA-3', tMet(*CAU*)E_Rev 5'-CGCTTAGC-CAACTTGAAAGAA-3', tVal(*CAC*)D_For 5'-GGGGAGCACAATGAAA-CAAAGG-3', tVal(*CAC*)D_Rev 5'-GGTGCAGCAAGTCTCAACTTTAC-3', ChrV_For 5′-CTGTCAGAATATGGGGCCGTAG-3′ and ChrV_Rev 5′-CCA-TACCCTC GGGTCAAACAC-3′. Primer sequences for tRNA Leu and tRNA Phe were described in Materials and Methods: Labeling and purifying newly synthesized RNA. Quantitative PCR was performed on the Roche LightCycler 480 instrument. Details of the PCR program and reactions were described previously [29]. Occupancies of Tfc3 and C160 at tRNA genes were calculated by determining the immunoprecipitation efficiency that is the amount of PCR product in the immunoprecipitated sample divided by the amount of PCR product in the input sample multiplied by 100. Occupancy values were normalized by subtraction of occupancy on an untranscribed fragment of chromosome V (ARS504) which served as a negative control.

3. Results

3.1. Primary tRNA transcripts accumulate in rsp5 and maf1 mutants under different environmental conditions

Inactivation of the global repressor of tRNA transcription Maf1 leads to the accumulation of primary tRNA transcripts during respiratory growth and under various stress conditions [7–10,39]. A similar molecular phenotype with higher levels of primary tRNA transcripts was observed for Rsp5 inactivation upon prolonged incubation at restrictive temperature [22,40]. We conducted Northern blot analysis to compare the accumulation of primary tRNA transcripts during Rsp5 and Maf1 inactivation under stress conditions that repress Pol III transcription, after transition of the strains from fermentation to respiratory growth, and incubation at elevated temperature [7]. We used two different temperature-sensitive *rsp5* alleles (*rsp5-1* [L733S] mutant that is defective in the catalytic HECT domain and *rsp5-19* [P418L] mutant that is defective in the WW3 domain that is responsible for the Rsp5 interaction with substrates) and a *maf1*Δ mutant with inactivation of the *MAF1* gene. Wild-type and mutant strains were grown to the exponential phase in glucose (YPD) medium at 30 °C and then transferred to a glycerol (YPGly; non-fermentable carbon source) medium at 37Z °C for 2 h (Fig. 1A, B). We observed a drop of primary transcripts for the wild-type strain but a significant increase for the *maf1*Δ mutant after the shift to YPGly medium at 37 °C for 2 h (Fig. 1A, B). The ratio of the primary transcript was higher (~2-fold) in the *maf1*Δ mutant compared with wild-type cells under respiratory growth conditions (Fig. 1B), thus confirming previously reported results [7,29]. In contrast, 4 h of incubation at 37 °C in YPD medium had no significant effect on levels of the primary tRNA transcript of the wild-type strain or *maf1*Δ mutant but resulted in a marked increase in primary tRNA transcripts for both *rsp5* mutants and did not change the levels of the mature form of tRNA (Fig. 1C, Fig. S1A, B). The statistically significant increase was 1.5- to 3-fold for the different tRNA probes that were tested in the *rsp5-1* mutant and 1.6- to 3.4-fold for the *rsp5-19* mutant (Fig. 1D) compared with the wild-type strain. Altogether, *rsp5* mutants accumulated primary tRNA transcripts under heat stress conditions but not under respiratory stress conditions, unlike the *maf1*Δ strain. This observation indicates different responses of the investigated strains to stress conditions.

3.2. Overexpression of the RNase P component RPR1 decreased pre-tRNA levels in rsp5 mutants under heat stress conditions

The first step in the maturation of most pre-tRNAs is cleavage of the 5′ end by endonuclease RNase P [41]. One exception is pre-tRNA Trp, for which 3′ maturation occurs before 5′ cleavage. This may indicate that another factor binds to pre-tRNA Trp and actively promotes its 3′ cleavage or that this is an intrinsic property that results from the structure of the pre-tRNA [42]. Major forms of precursors of tRNA Lys, tRNA Leu, and tRNA Tyr that accumulate in *rsp5* mutants during prolonged incubation at elevated temperature contain both unprocessed 5′ and 3′ ends. For pre-tRNA Trp, we observed the accumulation of 3′ and 5′-unprocessed forms and also 5′-unprocessed and 3′-processed forms (Fig. 1). These observations are consistent with an effect of Rsp5 on RNase P function at elevated temperature. To explore the effect of RNase P on tRNA processing in *rsp5* mutants, Northern blotting was again applied to analyze pre-tRNA levels in the wild-type strain and the *rsp5–1* and *rsp5–19* mutants after overexpression of *RPR1* encoding a catalytic RNA subunit of RNase P (Fig. 2). Under restrictive conditions, *RPR1* overexpression in both *rsp5* mutants resulted in an approximately 2-fold reduction of the amount of 3′- and 5′-unprocessed forms of all tested pre-tRNA and 5′-unprocessed form of pre-tRNA Trp compared with *rsp5* mutants with an empty plasmid. In contrast, the level of precursor tRNAs in the wild-type strain under restrictive conditions did not change after *RPR1* overexpression, as well as the levels of the mature form of tRNA (Fig. S1C). Next, the same blot was hybridized with the *RPR1* probe which is homologous to the fragment localized between nucleotides 84 and 113 of *RPR1* RNA. As predicted, this probe recognizes: mature *RPR1* (369 nucleotides), *RPR1* precursor (above 450 nucleotides) containing an extra 5′ leader (84 nucleotides) and a 3′ trailer (up to 33 nucleotides) (according to [43]). Unexpectedly, an additional form of *RPR1* migrating over 260 nucleotides was detected in *rsp5* mutants incubated at higher temperature (Fig. 2A, marked with an asterisk). We speculate that *RPR1* is cleaved into fragments by an unknown ribonuclease whose activity is controlled by Rsp5. These results suggest the role of Rsp5 ligase in the biosynthesis of the *RPR1* catalytic subunit of RNase P and, consequently, in the initial step of pre-tRNA maturation.

3.3. Newly synthesized tRNAs are reduced in rsp5 mutants under restrictive conditions

The accumulation of primary tRNA transcripts in the *rsp5* mutants could be the result of both inefficient tRNA maturation and an increase in tRNA transcription. To examine the impact of Rsp5 in tRNA transcription, levels of newly synthesized tRNAs were determined by in vivo labeling with 4-thiouracil (4tU), which is incorporated into the nascent RNA chain [32,36]. For efficient uracil importation, wild-type and *rsp5* mutant cells were transformed with a plasmid that encoded the permease gene, *FUI1*. After growth at a non-restrictive temperature of 30 °C to the exponential phase in glucose medium and then a restrictive temperature of 37 °C for 4 h, cells were incubated for 10 min with 4tU. Thiolated RNA was biotinylated and purified on streptavidin beads. 4tU-purified fractions were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) with primers that spanned transcription regions of the intron-containing tRNA Leu and tRNA Phe and intron-less tRNA His genes (Fig. 3). Upon the shift of wild-type cells to a restrictive temperature, tRNA transcription decreased. Levels of newly synthesized tRNA dropped from 53 % to 70 % compared with the same strain that was incubated at a non-restrictive temperature. Surprisingly, a stronger decrease in the synthesis of new tRNAs was observed for *rsp5* mutants; the levels of tRNA Leu, tRNA Phe, and tRNA His markedly decreased by around 72–93 % compared with wild-type in the same conditions, suggesting a role for Rsp5 in the control of tRNA transcription during heat stress. Thus, under prolonged incubation at high temperature, wild-type Rsp5 is required to control tRNA transcription.

3.4. The Tfc3 subunit of Pol III transcription factor interacts with Rsp5 ligase and is ubiquitinated

Next, we explored the possible role of Rsp5 ligase in the control of tRNA transcription by Pol III. Rsp5 ubiquitinates target proteins through direct or indirect interactions with them [44,45]. Rsp5 contains WW domains that mediate protein-protein interactions and recognize proline-rich sequences, called PY motifs [24]. Global studies identified several subunits of Pol III machinery and its general factors, TFIIIB and TFIIIC, as substrates for Rsp5 [24,25,46]. Additionally, one of them, the Tfc3 subunit of the TFIIIC transcription factor, contains a potential Rsp5-recognition PPLXXY motif in its C-terminal sequence. To verify the binding of Rsp5 with Tfc3, we used immunoprecipitation (IP). The wild-type strain and *rsp5* mutants that encoded TAP-tagged Tfc3 and the untagged wild-type strain were grown in YPD medium at 30 °C to the exponential phase. Total extract was immunoprecipitated with magnetic beads that were coated with anti-Tfc3 antibody or pre-immunoserum (Fig. 4A, anti-Tfc3 IP panels, lines 2–4, and NC, lines 1). Immunoprecipitation was confirmed by Western blot with anti-TAP antibody (Fig. 4A, upper panel) or anti-Tfc3 antibody (Fig. 4A, lower panel). The co-purification of Rsp5 was documented by Western blot with anti-Rsp5 antibody (Fig. 4A). The association of Tfc3 with Rsp5 was clearly visible for wild-type and the *rsp5–1* mutant but decreased in the *rsp5–19* mutant that was defective in substrate recognition by the WW3 domain of Rsp5 (Fig. 4A). Moreover, our additional IP studies using a wild-type strain not tagged with TAP epitope Tfc3 confirmed the interaction between

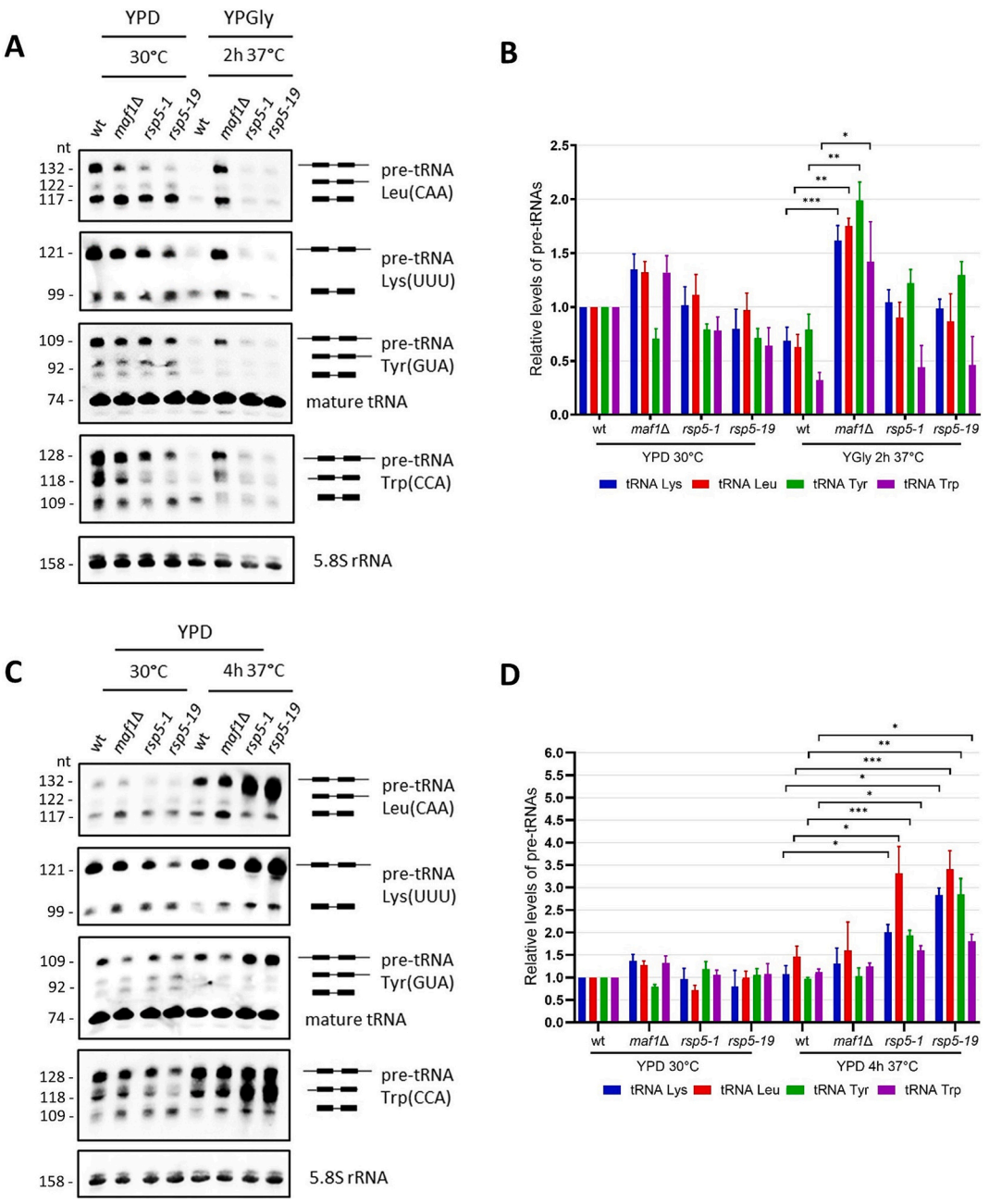

Fig. 1. *rsp5* and *maf1* mutants accumulate tRNA precursors in response to different environmental growth conditions. Wild-type (wt), *rsp5-1*, *rsp5-19*, and *maf1*Δ strains were grown in glucose (YPD) medium at 30 °C, harvested in the exponential phase, and transferred to glycerol (YPGly) medium for 2 h at 37 °C (A) or to an elevated temperature for 4 h at 37 °C (C). RNA was analyzed by Northern blot using probes that were specific to tRNA Lys(UUU), tRNA Leu(CAA), tRNA Tyr(GUA), and tRNA Trp(CCA). —▬▬—: tRNA precursor that contains introns and unprocessed 5′ and 3′ends; ▬▬ or —▬▬: tRNA precursor with intron and unprocessed 3′ or 5′ end; ▬▬: end-matured intron-containing pre-tRNA; mature tRNA. 5.8*S* rRNA served as the loading control. Nucleotide sizes (nt) of the corresponding forms of RNA were shown on the left. (B, D) Amounts of primary transcripts of tRNAs Lys, Leu, and Tyr (—▬▬—) and primary transcript together with 3′-end processed precursor of tRNA Trp (—▬▬— and ▬▬—) were normalized to the loading control (5.8*S* RNA) and calculated relative to amounts in the wt strain during growth in YPD medium to the exponential phase at 30 °C, which were set to 1. The figure shows the quantification of tRNA precursors during the transition of cells from the exponential phase (YPD) to the YPGly medium for 2 h at 37 °C (B) and elevated temperature for 4 h at 37 °C (D). The increased levels of pre-tRNA Lys, pre-tRNA Leu and pre-tRNA Trp in the *maf1*Δ strain compared to the wt strain grown on YPD at 30 °C were statistically significant ($p < 0.05$). Band intensities of Northern blot images were quantified by ImageJ. The data are expressed as the mean and standard deviation of three independent experiments. ***$p <$ 0.005, **$p < 0.01$, *$p < 0.05$, compared with wt strain grown under same conditions (two-tailed *t*-test).

Rsp5 and Tfc3 in a TAP epitope-independent manner (Fig. S2).

Additionally, a similar decrease in the Rsp5-Tfc3 interaction was observed by IP analysis by applying the *tfc3*Δ[*TFC3-TAP*] strain that expressed *HA-rsp5–19* from a plasmid (Fig. 4B). In this case, the magnetic beads were coated with an anti-HA antibody, and HA-tagged wild-type or the mutated version of *RSP5* was immunoprecipitated. The mutation of *rsp5–19* in the WW3 substrate-binding domain of Rsp5 resulted in an approximately 80 % reduction of the interaction between Rps5 and Tfc3. These results suggest that the WW3 domain is important for Tfc3-Rsp5 ligase interaction.

Tfc3 protein contains eight potential ubiquitination sites that have not been experimentally validated [47]. Therefore, we tested whether

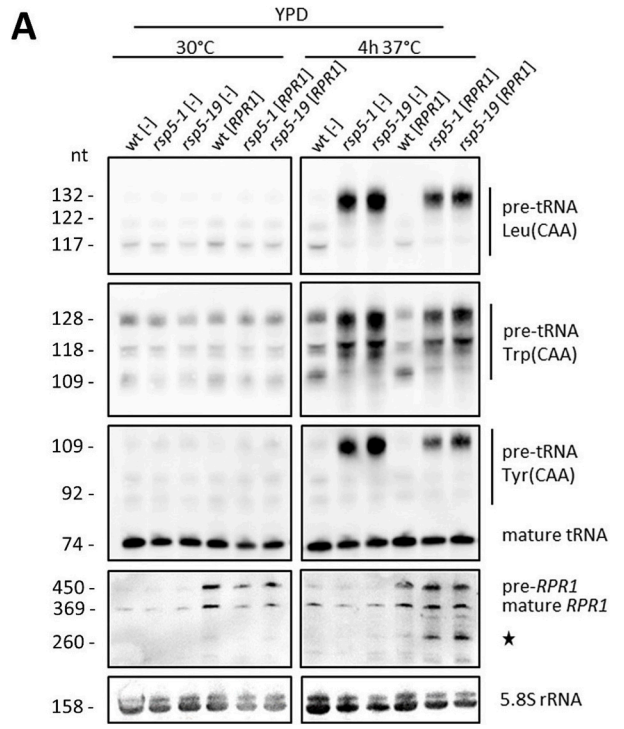

Fig. 2. Overexpression of *RPR1* partially reduces the accumulation of primary transcripts of tRNAs in *rsp5* mutants under restrictive conditions. Wild-type (wt), *rsp5–1*, and *rsp5–19* strains bearing *RPR1* from a multi-copy plasmid, [*RPR1*], or an empty plasmid, [−], were grown overnight in selective medium at 30 °C and reinoculated into YPD media to the exponential phase and shifted to 37 °C for 4 h. (A) RNA was analyzed by Northern blot as described in the legend to Fig. 1 using probes that were specific to tRNA Leu(CAA), tRNA Trp(CCA), tRNA Tyr(GUA) and *RPR1* RNA. The asterisk denotes an additional form of the *RPR1* transcript. (B) Quantification of pre-tRNA was done as described in the legend to Fig. 1. The data are expressed as the mean and standard deviation of three independent experiments. ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$, calculated for [*RPR1*]/[−] ratios in *rsp5–1* and *rsp5–19* under restrictive conditions (two-tailed *t*-test).

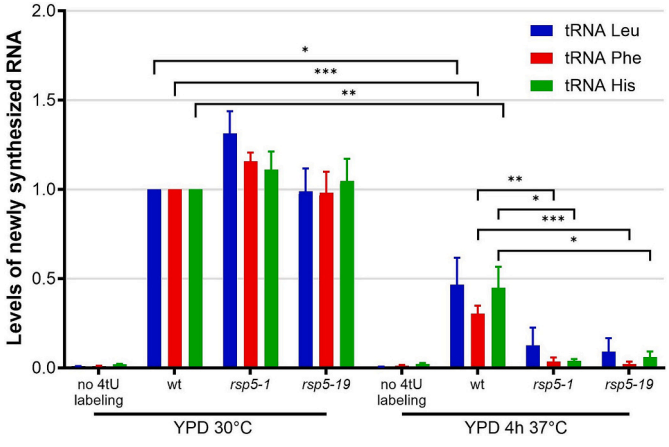

Fig. 3. Levels of newly synthesized tRNAs decreased in *rsp5* mutants after prolonged incubation at restrictive temperature. Wild-type (wt), *rsp5–1*, and *rsp5–19* strains bearing *FUI1* permease from a multi-copy plasmid that was grown overnight in selective medium at 30 °C were reinoculated into YPD media to the exponential phase and shifted to 37 °C for 4 h. Both cultures, before and after the shift to 37 °C, were then incubated with 4-thiouracil (4tU) for 10 min. 4tU-purified fractions were quantified by real-time PCR with primers that were specific for tRNA Leu, tRNA Phe, and tRNA His. A sample without 4tU labeling was analyzed and served as a negative control. All values were normalized to input RNA concentrations and to *S. pombe* spike-in (total RNA extracted from *S. pombe* grown to the exponential phase in YPD medium at 30 °C and labeled with 4tU for 10 min) levels of SpACT1 mRNA encoding actin of *S. pombe*. The wild-type strain under standard conditions (YPD medium) was set to 1. Bars represent the mean and standard deviation of three independent experiments. ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$, calculated for newly synthesized tRNAs (two-tailed *t*-test).

the Tfc3 protein is ubiquitinated in vivo. Wild-type cells that produced TAP epitope-tagged Tfc3 and His-ubiquitin from the plasmid with a gene under the copper ion inducible *CUP1* promoter were grown at 30 °C in selective medium to the exponential growth phase and incubated with copper sulfate for 3 h to induce His-ubiquitin. After cell lysis under denaturating conditions, proteins that were modified with His-ubiquitin were purified by using Ni-NTA Superflow resin. Aliquots of total proteins from the lysate (INPUT) and His-ubiquitin fraction that were eluted from the Ni-NTA resin (Ni-NTA pull down) were resolved by electrophoresis. An isogenic strain that produced Tfc3-TAP and was transformed with an empty vector was used as a negative control for Tfc3 ubiquitination (Fig. 5A). Tfc3 ubiquitination was assessed by Western blot analysis of the eluted fraction of ubiquitinated proteins with TAP-specific antibody to identify Tfc3-TAP protein (migrating around 150 kDa) and above migrated additional band (Fig. 5A, upper panel). Anti-Ubi antibody was used as a control for this fraction (Fig. 5A, lower panel). Modified forms of Tfc3 protein were also detected by the purification of Tfc3-TAP protein using magnetic beads that were coated with a Tfc3-specific antibody from wild-type cell lysates that contained a plasmid that encoded His-ubiquitin under non-denaturating conditions (Fig. 5B). Furthermore, despite the reduction of Tfc3 protein levels under restrictive conditions (YPD for 4 h at 37 °C), modified forms of Tfc3 were still observed (Fig. 5B).

The significant reduction of Tfc3 levels after the shift of wild-type cells to the elevated temperature suggested Tfc3 degradation. Therefore, we evaluated the effect of *rsp5* mutations on total levels of Tfc3 protein under restrictive conditions (Fig. S3). Wild-type, *rsp5–1*, and *rsp5–19* strains that produced Tfc3-TAP were transformed with a plasmid that encoded His-ubiquitin or an empty vector and grown to the exponential phase at 30 °C supplemented with 100 μM copper sulfate to express His-ubiquitin and then shifted to 37 °C for 4 h. Western blot analysis using anti-TAP antibody showed that the level of Tfc3 protein

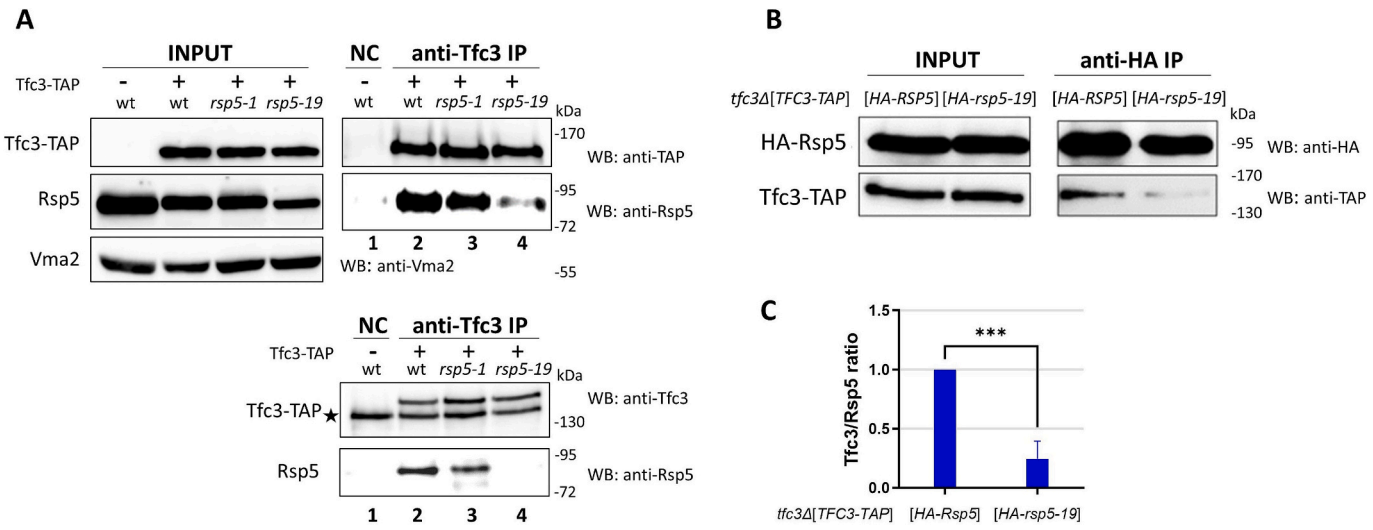

Fig. 4. Rsp5 interacts with Tfc3 via the WW3 domain. (A) Wild-type (wt) strain with or without Tfc3-TAP fusion and *rsp5* mutants that encoded Tfc3-TAP were grown at 30 °C to the exponential phase in glucose-rich medium. The total extract (INPUT) was immunoprecipitated with beads that were coated with pre-immune serum as a negative control (NC; first line) and anti-Tfc3 antibody (anti-Tfc3 IP; upper and lower panels, lines 2–4). The upper and lower IP panels show two independent IP experiments, where the blots were incubated with anti-TAP or anti-Rsp5 antibody (upper panel, WB: anti-TAP, WB: anti-Rsp5) or anti-Tfc3 or anti-Rsp5 antibody (lower panel, WB: anti-Tfc3, WB: anti-Rsp5) antibodies. The asterisk on the lower IP panel indicates a nonspecific band from the anti-Tfc3 antibody. Vma2 was used as a loading control for INPUT. (B) Total extracts that were isolated from *tfc3*Δ[*TFC3-TAP*] that expressed wild-type or the mutated version of *RSP5* with N-terminal HA fusion from plasmid, *HA-RSP5* or *HA-rsp5–19*, respectively, were immunoprecipitated with beads that were coated with anti-HA antibody (anti-HA IP). Purified proteins were visualized by incubation with anti-HA and anti-TAP antibodies. Molecular mass markers are displayed on the right. (C) Subunit ratios of Tfc3/Rsp5 IP fractions were calculated and refer to levels of respective subunits in the wild-type strain, which was set to 1. Bars represent the mean of three independent experiments. ***$p < 0.005$, calculated as wt/*rsp5–19* ratios (two-tailed *t*-test).

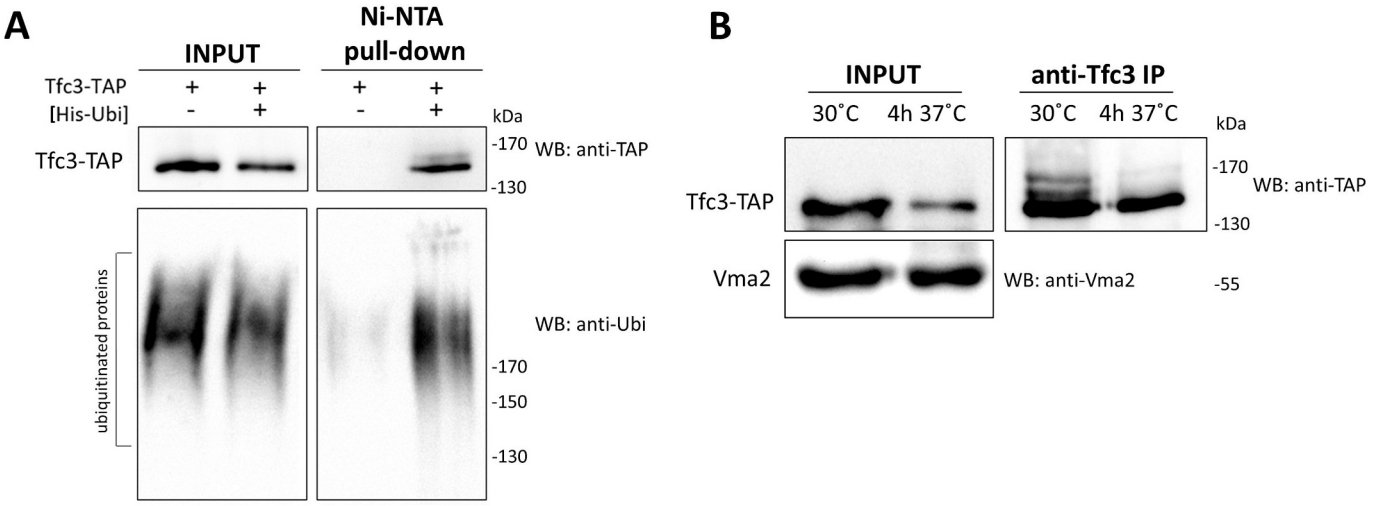

Fig. 5. Ubiquitination of Tfc3. Wild-type cells that produced Tfc3-TAP and His-ubiquitin (His-Ubi) under the *CUP1* promoter were grown at 30 °C to the exponential phase (A, B) or shifted to 37 °C for 4 h (B) and induced for 3 h with 100 μM copper sulfate to express His-Ubi. Wild-type Tfc3-TAP cells without overexpression of His-Ubi were treated with copper, as a negative control for ubiquitination (A). Ubiquitin-modified proteins were isolated with Ni-NTA beads (A). Tfc3-ubiquitinated protein was purified using magnetic beads that were coated with a Tfc3-specific antibody (B). Ubiquitinated forms of Tfc3-TAP were detected by Western blot with anti-TAP antibody. Anti-Ub antibody was used as a control of purified ubiquitinated proteins on a separate second blot (A, lower panel). Vma2 served as the loading control (B). Molecular mass markers are shown on the right.

was similar in wild-type and *rsp5* mutants when the cells were grown at 30 °C and 37 °C (Fig. S3A). Interestingly, cooper treatment-induced overexpression of His-ubiquitin during the shift of cells to the elevated temperature significantly decreased Tfc3 levels independently of Rsp5 (Fig. S3B, C). This suggests that Tfc3 degradation at the higher temperature was independent of ubiquitination by Rsp5.

3.5. Weakened TFIIIC occupancy of tRNA genes and the interaction between TFIIIB and TFIIIC in the rsp5-1 mutant are inversely correlated with Pol III occupancy

Knowing that Tfc3 is ubiquitinated and interacts with Rsp5, we investigated the role of Rsp5 ligase in the regulation of Pol III transcription machinery by the TFIIIC transcription factor. The transcription of tRNA genes in response to favorable growth conditions (i.e., growth of cells in glucose medium) involves the dissociation of TFIIIC from tRNA

genes [29]. Therefore, we tested whether Rsp5 influences TFIIIC dissociation from tRNA genes. We analyzed the occupancy of tRNA genes by the Tfc3 subunit of the TFIIIC transcription factor using a chromatin IP approach. Wild-type and *rsp5-1* cells that exhibited ubiquitinated forms of Tfc3 protein harbored the Tfc3-TAP-tagged epitope. The efficiency of Tfc3-TAP immunopurification greatly increases by using a Tfc3-specific antibody [29]. Thus, tDNA fragments were immunopurified with Tfc3 antibody and quantified by qRT-PCR with primers that spanned transcription regions of the intron-containing tRNA Leu and tRNA Phe genes and intron-less tRNA Met and tRNA Val genes. The *rsp5-1* mutation significantly decreased TFIIIC occupancy of all tested tRNA genes by ~2-fold (Fig. 6A). The decrease in TFIIIC occupancy in the absence of Rsp5 catalytic activity in the *rsp5-1* mutant supports a role for Rsp5 in the regulation of tRNA transcription machinery.

To further understand the mechanism of action of Rsp5 in regulating the tRNA transcription apparatus, we analyzed the effect of Rsp5 on interactions between TFIIIC and TFIIIB transcription factors. The dissociation of TFIIIC from tRNA genes under favorable growth conditions (i.e., YPD medium) correlates with a decrease in the interaction between TFIIIB and TFIIIC [29]. Thus, we compared the interaction between chromatin-associated TFIIIB and TFIIIC in the wild-type strain and *rsp5-1* mutant by co-IP. The tested strains encoded green fluorescent protein (GFP)-tagged Tfc4 (the representative TFIIIC subunit) and HA-tagged Bdp1 (the TFIIIB subunit). The untagged wild-type strain was used as a control. Using magnetic beads that were coated with GFP-specific antibody, Tfc4-GFP was immunoprecipitated, and interactions with TFIIIB subunits, Bdp1 and Brf1, were examined by co-IP (Fig. 6B). Co-purified proteins were subjected to quantitative Western blot analysis. Associations between Tfc4 and Bdp1/Brf1 clearly decreased ~2-fold in *rsp5–1* mutant cells compared with the wild-type strain (Fig. 6B, C), indicating that *rsp5–1* weakens the interaction between TFIIIB and TFIIIC. Decreased TFIIIB-TFIIIC interaction is correlated with lower occupancy of the tRNA gene by TFIIIC in the *rps5* mutant, revealing Rsp5-dependent regulation of the Pol III machinery by TFIIIC.

The correlation of TFIIIB-TFIIIC interaction with occupancy of the tRNA gene by TFIIIC is in agreement with a model of Pol III regulation by TFIIIC, which functions in activation and repression of tRNA gene transcription [29]. This model involves the inverse correlation of TFIIIC occupancy with Pol III occupancy. We thus analyzed the effect of *rsp5–1* mutant on the occupancy of tRNA genes by Pol III enzyme. The HA-

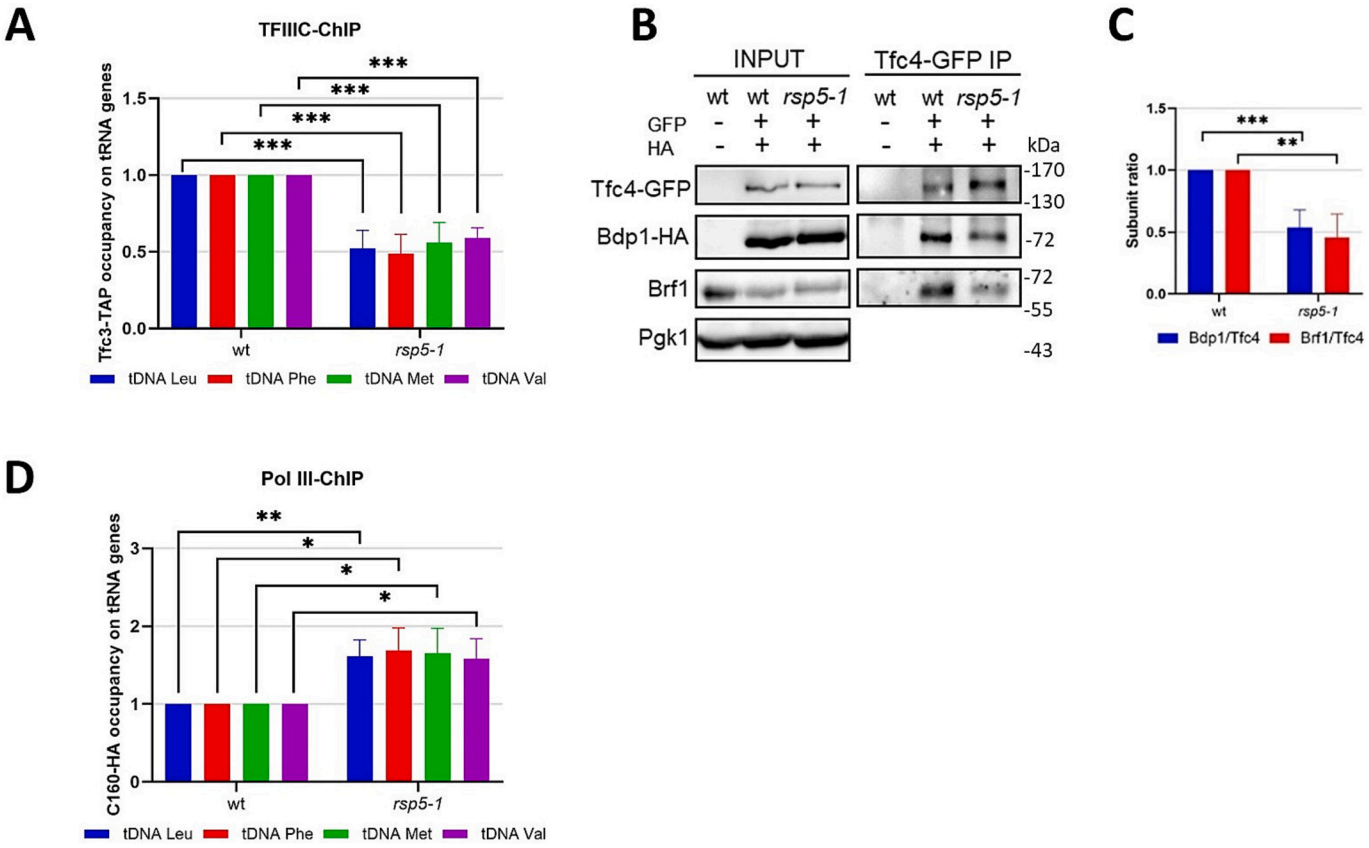

Fig. 6. Inactivation of the catalytic activity of Rsp5 influences recruitment of TFIIIC and Pol III to tRNA genes and decreases TFIIIB-TFIIIC interaction. (A) Wild-type (wt) and the isogenic *rsp5–1* strain that produced Tfc3-TAP were grown in glucose (YPD) medium at 30 °C to the exponential phase. Cross-linked chromatin was immunoprecipitated with an antibody against Tfc3, followed by qRT-PCR with primers that were specific to single tDNAs: intron-containing tL(*CAA*)G1 and tF(*GAA*)H1 (designated tDNA Leu and tDNA Phe) and intron-less tM(*CAU*)E and tV(*CAC*)D (designated tDNA Met and tDNA Val). As a control, we used primers that were homologous to a region that is not occupied by Pol III (ChrV). Occupancy, calculated as a percentage of IP over input chromatin, was set to 1 for the wt strain. The data are expressed as the mean and standard deviation of three independent experiments. ***p value < 0.005, calculated for Tfc3-TAP occupancy for all tested tDNAs as wt/*rsp5-1* ratios (two-tailed t-test). (B) Cross-linked chromatin extracts that were isolated from untagged wild-type (wt) and *rsp5-1* strains that produced the double GFP-tagged Tfc4 subunit of TFIIIC and HA-tagged Bdp1 subunit of TFIIIB that were grown in glucose medium at 30°C to the exponential phase were subjected to IP with magnetic beads that were coated with an anti-GFP antibody, followed by the elution of bound proteins. Immunopurified proteins were analyzed by SDS-PAGE and Western blot using anti-GFP, anti-HA, and anti-Brf1 antibodies. Pgk1 was used as the loading control of the total cellular extract (INPUT). Molecular mass markers are displayed on the right. Band intensities on the Western blot images were quantified by ImageJ. (C) Bdp1/Tfc4 and Brf1/Tfc4 ratios were calculated. The ratio in the wt strain was set to 1. The data are expressed as the mean and standard deviation of three independent experiments. ***$p < 0.005$, **$p < 0.01$ calculated as wt/*rsp5–1* ratios (two-tailed t-test). (D) Wild-type and isogenic *rsp5–1* strain that expressed C160-HA were grown in YPD medium at 30 °C to the exponential phase. Cross-linked chromatin was immunoprecipitated with antibodies against HA, followed by qRT-PCR with primers that were specific to single tDNAs as specified above.

tagged largest Pol III subunit, C160, was used as bait. tDNA fragments were immunopurified with HA antibodies and quantified by qRT-PCR with primers that spanned transcription regions of the intron-containing tRNA Leu and tRNA Phe genes, as well as intron-less tRNA Met and tRNA Val genes. The *rsp5–1* mutation significantly increased Pol III occupancy on all tested tRNA genes by ~1.6-fold (Fig. 6D). Indeed, the increase in Pol III occupancy in the absence of Rsp5 catalytic activity in the *rsp5–1* mutant is inversely correlated with the decrease in TFIIIC occupancy.

4. Discussion

In the present study, we revealed the role of Rsp5 ubiquitin ligase in the control of both, tRNA transcription and processing. We combined Northern blot analysis and in vivo labeling, showing that the accumulation of primary tRNA transcripts in *rsp5* mutants at an elevated temperature indeed resulted from a defect in tRNA processing but was also associated with a decrease in newly synthesized tRNAs. Thus, the effects of Rsp5 on tRNA transcription and processing under stress conditions appear to be opposite.

Various examples of an interplay between tRNA transcription and processing have been reported previously. The accumulation of primary tRNA transcripts in a *maf1*Δ mutant that was grown under stress conditions resulted from the lack of Pol III repression, consistent with the essential role of Maf1 as a negative regulator of tRNA transcription [7,48]. As a consequence of saturating tRNA maturation machinery with higher amounts of primary transcripts, tRNA processing and nuclear export are inhibited in *maf1*Δ cells [49]. The correlation between Pol III transcription and tRNA processing is a function of Rpc11, a subunit of *S. pombe* Pol III, which plays a role in the termination of tRNA transcription and 3' tRNA cleavage, limiting the length of 3′-oligo(U) and thus significantly restricting pre-tRNAs to a La-independent pathway of maturation [50]. Another study identified the *RPR1* gene, which encodes a catalytic subunit of RNase P, as a suppressor of missense mutation in the Tfc3 subunit of TFIIIC [33] and deletion in the sequence encoding Bdp1 subunit of TFIIIB [51]. The *RPR1* gene is transcribed by RNA Pol III, and its amount is reduced by mutations of Pol III factors. A defect in pre-tRNA synthesis for the *tfc3* or *bdp1* mutant, combined with low RNase P levels, would lead to mature tRNA levels that are incompatible with normal cell growth. Increasing *RPR1* expression via gene dosage likely optimizes RNase P assembly and thus tRNA maturation [33]. Human RNase P plays a direct role in Pol III transcription through an association with Pol III and the chromatin of active tRNA and 5*S* rRNA genes [52]. The knockdown of human RNase P abolished the assembly of initiation complexes by preventing formation of the initiation complex of Pol III [53]. A molecular model was presented showing human Pol III initiation complexes, which are assembled on tRNA genes and process precursor transcripts to mature forms [54]. These results indicate a dual role for RNase P in the maturation of tRNA and initiation of Pol III transcription.

Based on the data reported herein, the relationship between tRNA transcription and processing by RNase P involves the activity of Rsp5, the ubiquitin ligase. Rsp5, a conserved E3 ubiquitin ligase of the NEDD4 family, regulates multiple processes, including control of Pol II transcription, endocytosis, lipid biosynthesis, multivesicular body sorting, actin cytoskeleton organization, and protein aggregate degradation, among others (for review, see [18–20]). Our data reveal a novel function of Rsp5 in the regulation of tRNA transcription by Pol III. We found that catalytically inactive Rsp5 reduced the recruitment of TFIIIC to tRNA genes and the interaction between TFIIIC and TFIIIB transcription factors, which is inversely correlated with Pol III occupancy. Our data are consistent with a model of Pol III regulation by TFIIIC, which functions in activation and repression of tRNA gene transcription [29]. Moreover, we have shown that Tfc3 subunit of TFIIIC interacts with Rsp5 and is ubiquitinated. Based on these results, we propose a molecular mechanism in which the ubiquitination of Tfc3 in an Rsp5-dependent manner affects recruitment of Pol III machinery to tRNA genes and TFIIIB-TFIIIC interactions.

The present study also found that *rsp5* mutations at restrictive temperature prevented 5′-end cleavage of the tRNA primary transcript. Thus, Rsp5 is potentially involved in the early step of tRNA processing. The role of Rsp5 in tRNA processing was suggested previously but not proven experimentally. The prolonged incubation of *rsp5–3* mutant cells at an elevated temperature resulted in an increase in primary tRNA transcripts and the nuclear accumulation of tRNA [21]. Other studies reported the nuclear accumulation of tRNA in the *rsp5–13* mutant and an increase in tRNA precursors in *rsp5-T761D* and *rsp5–1* mutants under conditions of a shift to a restrictive temperature [31,40].

Present work sheds new light on the role of Rsp5 in the initial step of tRNA maturation involving RNase P. 5′-End processing is the first step of pre-tRNA maturation for every tRNA, except pre-tRNA Trp, which is initially processed from the 3′ end. Here, we observed the accumulation of 3′- and 5′-unprocessed forms of primary tRNA transcripts in *rsp5* mutants under restrictive conditions. Significantly, for pre-tRNA Trp, we detected the accumulation of 3′-processed and 5′-unprocessed forms. Accumulation of these tRNA precursors was reduced by the overexpression of *RPR1*, a catalytic RNA subunit of RNase P. Noteworthy, biosynthesis of *RPR1* by itself seems to be dependent on Rsp5. Besides mature *RPR1* (369 nucleotides), *RPR1* precursor (above 450 nucleotides) the probe which we used for Northern blot recognizes an additional form of *RPR1* in *rsp5* mutants migrating over 260 nucleotides. One possible scenario is that *RPR1* RNA is cleaved by an unknown, Rsp5-dependent ribonuclease, resulting in at least two fragments of lengths around 260–300 nucleotides (recognized by the specific Northern probe) and 60–100 nucleotides (not recognized by the probe). Given the tertiary structure of the RNase P complex and secondary structure of *RPR1* RNA [55], it is clear that the cleavage of *RPR1* inactivates RNase P enzyme. Only the complete *RPR1* RNA can recognize the substrate, bind it in the correct position and carry out the catalytic function. Assuming that the *RPR1* RNA stability depends on Rsp5, the same degradation of *RPR1* RNA occurs in *rsp5* mutants at 37 °C when there is no overexpression of *RPR1*, but the level of truncated *RPR1* RNA is too low to detect. We consider the possibility that the truncated *RPR1* RNA formed in *rsp5* mutants at higher temperature titrates protein subunits of RNase P; although full-length *RPR1* is synthesized the assembly of the RNase P enzyme is incomplete, resulting in no growth. Overexpression of *RPR1* in the *rsp5* mutant will supply a higher level of uncleaved *RPR1* RNA, enabling suppression.

RPR1 is synthesized by Pol III, but its maturation/degradation, not described in the literature, possibly involves the Rsp5-modifying enzyme. In summary, our results suggest that a reason for the accumulation of tRNA precursors in *rsp5* mutants under restrictive conditions is a defective activity of *RPR1*, the catalytic subunit of RNase P.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2025.119993.

CRediT authorship contribution statement

Aleksandra Łopusińska: Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Michał Tys:** Investigation, Formal analysis, Data curation. **Magdalena Boguta:** Writing – review & editing, Formal analysis, Conceptualization. **Małgorzata Cieśla:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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