Research paper

Functional suppression of a yeast *maf1* deletion mutant by overdose of the N-terminal fragment of the largest RNA polymerase III subunit, C160

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ABSTRACT

Maf1 is a general and global negative regulator of RNA polymerase III (Pol III) transcription. Under repressive conditions, Maf1 binds directly to the Pol III complex and sequesters Pol III elements that are involved in transcription initiation. To further understand Pol III regulation, we searched for genetic bypass suppressors of a *maf1* deletion mutant (*maf1Δ*) of *Saccharomyces cerevisiae*. Strains that carried *maf1Δ* were temperature-sensitive on media that contained nonfermentable carbon sources and showed the antisuppressor phenotype. Suppressors allowed colonies to grow at the restrictive temperature on glycerol media and partially complemented the antisuppressor phenotype of *maf1Δ*. DNA plasmids that were identified as overdose suppressors encoded N-terminal fragments of the largest Pol III subunit, C160 of various lengths. The shortest fragment, 372 amino acids long, the overdose of which partially complemented the antisuppressor phenotype and temperature-sensitive respiratory growth of *maf1Δ*, was named C160-NTF. In this study, we showed that the expression of HA-tagged C160-NTF resulted in accumulation of approximately 40 kDa protein that was distributed throughout the yeast cell, in the cytoplasm and nucleus. The overdose of C160-NTF led to decrease of tRNA transcription in *maf1Δ* mutant cells, demonstrating functional suppression. Levels of newly synthesized individual tRNAs and Pol III occupancies on tRNA genes were decreased by C160-NTF in the *maf1Δ* mutant. Additionally, we analyzed the effect of C160-NTF overproduction and the presence of Maf1 on the associations among Pol III subunits. Previous structural analyzes of Pol III have indicated that the N-terminal region of C160 interacts with the C82-34-31 heterotrimeric Pol III subcomplex. We suggest that the negative effect of C160-NTF overdose on tRNA transcription is related to defective Pol III assembly, because overproduction of C160-NTF altered C160 interactions with C34 and C82 in the *maf1Δ* mutant.

1. Introduction

In all eukaryotes, nuclear DNA transcription is performed by at least three different RNA polymerases (Pols). Pol I and Pol II specialize in the synthesis of ribosomal RNA (rRNA) and messenger RNA (mRNA), respectively, whereas products of Pol III are small noncoding RNAs, mainly tRNAs. Pol III is the most complex enzyme of eukaryotic Pols and highly conserved between organisms. In the yeast *Saccharomyces cerevisiae,* Pol III is composed of 17 subunits, 10 of which comprise the structural core. C160 and C128 catalytic subunits form the active-center cleft, C11 is involved in transcription termination, AC40 and AC19 are common between Pol III and Pol I, and five small subunits are shared

among all three Pols. On the periphery of the core enzyme are additional subunits that form specific Pol III-specific subcomplexes. C82-C34-C31 functions in initiation, and C53-C37 functions in termination. Additionally, C17 and C25 form a Pol III stalk that is involved in transcription initiation (Hoffmann et al., 2015).

Pol III is negatively regulated by Maf1 protein, which is conserved from yeast to humans. Maf1 is not a typical DNA-binding transcription factor. Instead of binding to promoter elements, under repressive conditions, Maf1 binds directly to the Pol III complex (Pluta et al., 2001). Moreover, the Pol III-Maf1 association increases under unfavorable growth conditions and correlates with Maf1 dephosphorylation. Analyses of the Pol III structure in complex with Maf1 showed that Maf1

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binds to the Pol III clamp at the rim of the cleft and rearranges the structure of the C82-C34-C31 heterotrimer over the active center. By relocating a specific WH domain of the C34 subunit, Maf1 weakens the interaction between C34 and the Brf1 subunit of the TFIIIB initiation factor, thereby impairing Pol III recruitment to promoters. Finally, a cryo-electron microscopy structure of yeast Maf1 that binds to Pol III established that Maf1 sequesters Pol III elements that are involved in transcription initiation and binds the mobile C34 WH2 domain, sealing off the active site (Vorländer et al., 2020).

Interactions among Pol III subunits, subunit regions, and regulatory factors were originally identified by applying both traditional and molecular genetic approaches (Archambault and Friesen, 1993; Flores et al., 1999). Some dosage-dependent associations were identified by a screen of a yeast genomic library borne on a high-copy-number vector for the extragenic suppression of conditional mutations of genes that encode Pol III subunits (Lalo et al., 1993). Using the same approach, genetic interactions between Pol III and Maf1 were found. Genomic fragments that encode N-terminal regions of the C160 subunit of Pol III were cloned as multicopy suppressors that operate specifically on a mutation of the gene that encodes Maf1 (Boguta et al., 1997).

N-terminal fragment of the C160 (aa 1–245) binds directly Maf1 protein (Oficjalska-Pham et al., 2006). The C160 clamp domain located at the N-terminus contacts the C82-34-31 heterotrimeric Pol III subcomplex through the C82-WH1/WH4 and C34-WH3 winged-helix domains. Additionally, the clamp core region of C160 binds the N-terminal region of the C31, similar to the binding mode of homologous Rpc1 and Rpc7α in the human Pol III complex (Girbig et al., 2021; Shekhar et al., 2023). Furthermore, the interaction of the N-terminal part of C160 with the peripheral stalk Pol III subcomplex was demonstrated (Hoffmann et al., 2015). The C17-C25 stalk subunits are anchored to the core by extensions of the C160 subunit and a Pol III-specific C25 helix that contacts the clamp domain (Hoffmann et al., 2015).

Here, we document functional suppression of *maf1* deletion mutant, showing that an overdose of C160-NTF (the shortest fragment of the cloned C160) decreased Pol III occupancy on tRNA genes and led to lower tRNA transcription in the *maf1Δ* mutant. We suggest that this negative effect is related to defective Pol III assembly because C160-NTF sequesters other subunits and doesn't allow Pol III formation.

2. Materials and methods

2.1. Yeast strains and plasmids

The following yeast strains were used: MB159-4D (*MAT*a *SUP11 ade2-1 ura3-1 leu2-3,112 lys2-1 his3*) and the deletion mutant *maf1Δ* (*MAT*a *SUP11 ade2-1 ura3-1 leu2-3,112 lys2-1 his3 maf1::KanMX6*) (Kwapisz et al., 2002); YPH499 C160-HA (*MAT*a *ura3-52 his3-Δ200 ade2-101 trp1-Δ63 lys2-801 leu2-Δ1 C160::3HA::KanMX6*) (Soutourina et al., 2006) and the deletion mutant *maf1Δ* (*MAT*a *ura3-52 his3-Δ200 ade2-101 trp1-Δ63 lys2-801 leu2-Δ1 C160::3HA::KanMX6 maf1::URA3*) (Cieśla et al., 2018). Plasmids used in this study are: pFL44L (*URA3/2μ*) and pFL46L (*LEU2/2μ*) (Bonneaud et al., 1991); pFL44L-C160-NTF (*URA3/2μ*) (Boguta et al., 1997); pFL44L-C160-NTF-HA (Oficjalska-Pham et al., 2006); pFL46L-C160-NTF (*LEU2/2μ*) [constructed in this study by cutting out the C160-NTF fragment from the pFL44L vector by using SacI and SphI restriction enzymes and ligating it into the pFL46L vector digested with SacI and SphI enzymes].

2.2. Media and growth conditions

Yeast strains were grown in YPD (2 % glucose, 2 % peptone, 1 % yeast extract), YPGly (2 % glycerol, 2 % peptone, 1 % yeast extract) and SC (2 % glucose, 0.67 % yeast nitrogen base without amino acids) medium. SC-ura or SC-leu contained 20 g/ml of the amino acids required for growth, except for uracil or leucine, respectively. The start liquid cultures were grown overnight in SC-ura or SC-leu, transferred to YPD and grown to the exponential phase and then transferred to YPGly medium and incubated at 37 °C for 2 h. Solid media contained 2 % agar. All reagents used for media were Difco products.

2.3. Immunofluorescence

S. cerevisiae cells were fixed in culture by adding formaldehyde to a final concentration of 3.7 % for 1 h, spun down, washed and converted to spheroplasts, which were applied on microscopic slides covered with polylysine and next treated as described previously (Oficjalska-Pham et al., 2006). Anti- HA antibody (1:500 for 1 h) was added followed by secondary Cy3-conjugated anti-mouse antibody (1:250 for 1 h). Nuclei were stained with DAPI. A Carl Zeiss Axio ImagerM2 microscope equipped with a 100 × oil objective was used. Images were collected with the AxioVision 4.8 program.

2.4. Chromatin immunoprecipitation and quantitative PCR

Chromatin isolation and immunoprecipitation from the yeast cells expressing HA epitope-tagged C160 were performed as described previously (Cieśla et al., 2018). The input and immunoprecipitated samples were assayed by quantitative PCR to assess the extent of C160 protein occupancy at different genomic regions. The sequences of the primers used in this study were: tRNALeu(CAA)G1For 5′- AGAACCGAAACA-TACAAATAAGTGGT-3′; tRNALeu(CAA)G1Rev 5′- TGATCACAGAACC AAAAAGATAAAA-3′; tRNAPhe(GAA)H1For 5′-TCAGAATTTCAACAAA-TAGTAAGCGGA-3′; tRNAPhe(GAA)H1Rev 5′- TGCCCTGTCATGTTAG-GAAATACA-3′; tRNAMet(CAU)EFor 5′-GCGGACCTCAATATAAGCGA-3′; tRNAMet(CAU)ERev 5′- CGCTTAGCCAACTTGAAAGAA-3′; ChrVFor 5′-CTGTCAGAATATGGGGCCGTAG-3′ and ChrVRev 5′-CCATACCCTC GGGTCAAACAC-3′. Quantitative PCR was performed on Roche Light-Cycler 480 instrument. Details of the PCR program and reactions were described previously (Cieśla et al., 2018). Occupancies of 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 with occupancy on untranscribed fragment of chromosome V (ARS504) which served as a negative control.

2.5. Agarose gel and northern blotting

Total RNA was isolated, separated on a 2.8 % agarose gel, and stained with EtBr using equal amounts of RNA per lane (5 μg). RNA isolation and northern hybridization was done as described previously (Foretek et al., 2016). 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 gel onto a Hybond-N+membrane (Amersham) by electroblotting in 0.5 X TBE and crosslinked by UV radiation. The following DIG-labeled oligonucleotides were used for RNA hybridization: tRNA Leu(CAA) 5′-TATTCCCACAGTTAACTGCGGTCA-3′; tRNA Trp (CCA) 5′-GCAATCTTATTCCGTGGAATTTCCAAG-3′; tRNA Phe(GAA) 5′-GCGCTCTCCCAACTGAGCT-3′ and 5.8S rRNA, 5′-GCGTTGTTCATC-GATGC-3′.

2.6. Western blotting

Total cellular protein was extracted from yeast cells by alkaline lysis as described previously (Cieśla et al., 2007). Protein extracts were analyzed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) by western blotting with anti-HA at a 1:5000 (Covance), anti-C82 at a 1:5000, anti-C53 at a 1:20,000, and anti-C34 at a 1:5000 overnight at 4 °C. Antibodies specific for Pol III subunits were a gift from O. Lefebvre (CEA/Saclay, France). Then, nitrocellulose membranes were incubated with secondary anti-mouse or anti-rabbit

antibodies coupled to horseradish peroxidase (Dako) at a dilution of 1:5000 for 1 h and visualized by chemiluminescence using the ECL detection kit (Bio-Rad).

2.7. Immunoprecipitation of C160-HA

Extracts were prepared in IP buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.05 % NP-40, 0.5 mM ditiotreitol (DTT), 5 % glycerol, O-complete protease inhibitor; Roche) by mechanical disruption with beads as described before (Cieśla et al., 2015). Mouse Dynabeads IgG magnetic beads (50 μl) (Invitrogen), washed three times with 0.5 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS), were incubated overnight with equal amounts (2 mg) of protein extracts in IP buffer with gentle shaking at 4 °C. After incubation, beads were washed three times in IP buffer. Immunoprecipitated proteins were eluted by boiling for 5 min in SDS sample buffer and analyzed on 10 % SDS-PAGE gel.

3. Results

3.1. Genetic suppression of maf1Δ by overproduction of the N-terminal fragment of the Pol III C160 subunit

Despite its role as the sole negative regulator of Pol III transcription,

Maf1 is not essential in yeast. The control of tRNA synthesis, mediated by Maf1, becomes essential when yeast cells adapt to respiration in media with a non-fermentable carbon source (Cieśla et al., 2007). Growth of the *maf1Δ* mutant is compromised on a YPGly glycerol medium, especially at elevated temperature and it is correlated with accumulation of newly transcribed tRNAs. Moreover, lower efficiency of the tRNA suppressor *SUP11* (tRNA Tyr/UAA) in *maf1Δ* resulted in a red colony color in the *SUP11 ade2-1* genetic background, which is related to an effect of Maf1 on translation (Kwapisz et al., 2002). Both phenotypes of *maf1Δ* were suppressed by overdose of the N-terminal fragment of the 372-amino-acid (aa) C160 protein, hereinafter referred to as C160-NTF (Fig. 1A). Overexpression of the C160-NTF fragment has no effect on the growth phenotype of the wild type strain (Fig. S1).

C160, the largest catalytic subunit of Pol III, shares several homologous domains with the largest subunits of Pol I (A190) and Pol II (B220), but lost the C-terminal domain (CTD) that is present in B220 (Fig. 1B). C160-NTF contains the conserved zinc binding domain a, which is essential for the structural integrity of Pol III enzyme (Werner et al., 1992). The domain a of C160 has zinc binding properties itself when autonomously expressed in *Escherichia coli* (Werner et al., 1992).

Overexpression of the insert that encoded the HA-tagged C160-NTF fragment in yeast resulted in accumulation of approximately 40 kDa protein (Fig. 1C) that was distributed throughout the cell, in the cytoplasm and nucleus (Fig. 1D).
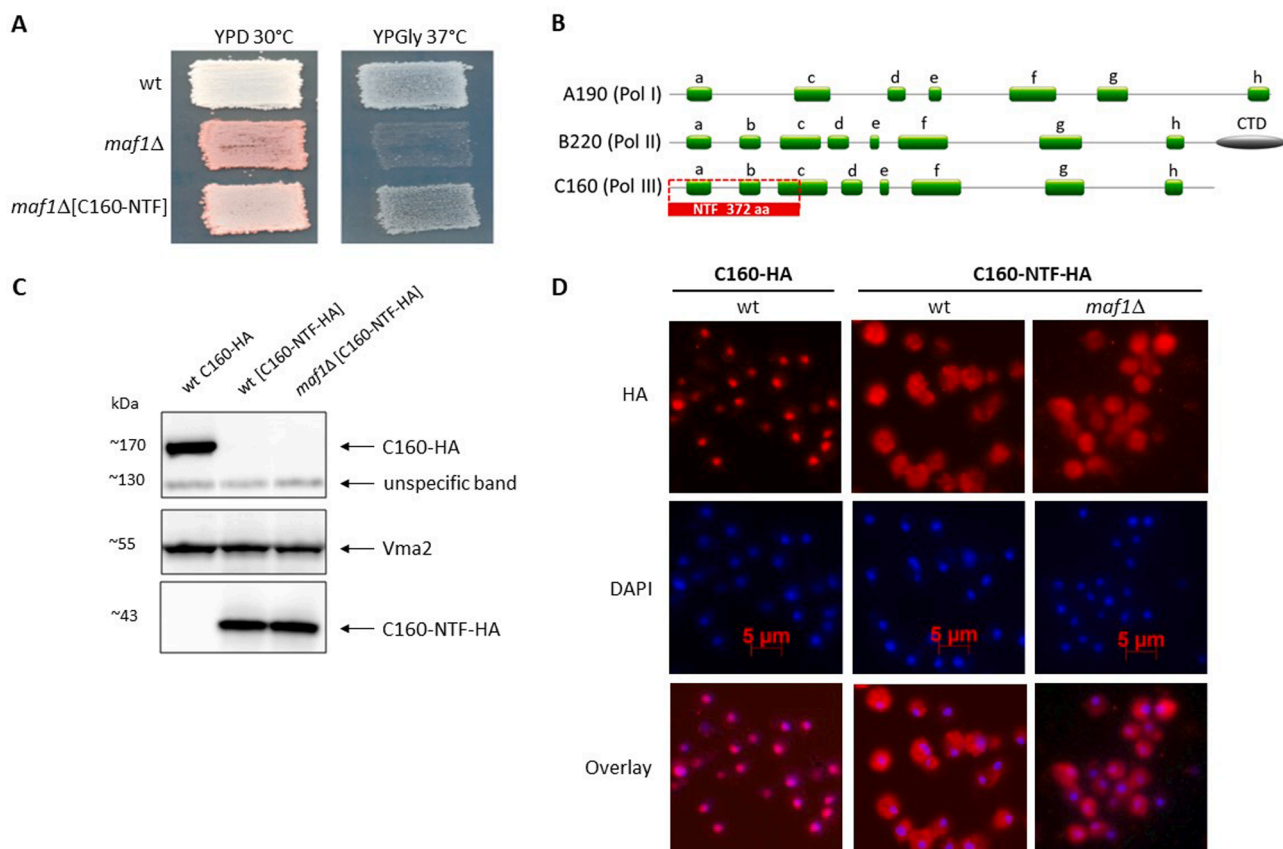
Fig. 1. (**A**) Suppression of *ade2-1* mutation and growth by C160-NTF on medium with glycerol were compared among the MB159-4D (wt) control strain and *maf1Δ* deletion mutant that was transformed with an empty vector or a plasmid that encoded the N-terminal fragment (372 aa) of the C160 subunit of Pol III, C160-NTF. Cells were grown on SC-ura overnight, replica-plated on YPD or YPGly, and incubated for 3 days at the indicated temperatures. (**B**) C160 shares regions of homology (a-h domains) with the largest subunit of RNA polymerase I and II of yeast. C160-NTF covers regions of homology of the C160 subunit that involve the a and b domains. (**C, D**) Cells were grown in minimal medium overnight, then transferred to glucose medium and harvested in the exponential phase. Yeast that expressed HA-tagged C160 or C160-NTF were analyzed by western blot (**C**) and immunofluorescence (**D**) with HA-specific antibody. Vacuolar membrane ATPase, Vma2 was used as a protein loading control (**C**). DAPI was used to stain nuclear DNA (**D**).

3.2. Overproduction of the N-terminal fragment of Pol III C160 subunit downregulates the Pol III transcription of tRNA genes in maf1Δ cells

Maf1 is required for the repression of Pol III transcription upon the transition of yeast between fermentation and aerobic respiration by utilizing non-fermentable carbon sources (Cieśla et al., 2007). A metabolic switch that activated Maf1 involved the transition of cells that were grown in glucose medium under optimal conditions (YPD) to a medium that contained glycerol (YPGly), a non-fermentable carbon source, and incubation at 37 °C for 2 h. This growth protocol, referred to as "YPD→YPGly," was applied to investigate the way in which C160-NTF overproduction affects Pol III transcription and whether this effect depends on Maf1 (Figs. 2 and 3).

A substantial decrease in the Pol III association with tRNA genes was observed upon the YPD→YPGly transition in wild type cells but not in the *maf1Δ* mutant, as observed previously (Cieśla et al., 2018). A partial decrease in Pol III occupancy in *maf1Δ* cells, however, was seen when C160-NTF was overproduced. Upon the YPD→YPGly transition, the Pol III association with tDNAs in *maf1Δ* cells was significantly different and was reduced by an average of 25 % when C160-NTF was overproduced. Notably, no significant effects of C160-NTF were observed in wild type cells (Fig. 2).

Changes in Pol III occupancy on tDNAs upon the YPD→YPGly transition correlated with alterations of *de-novo* transcription as indicated by the levels of primary transcripts and intron-containing tRNA precursors. A negative effect of C160-NTF on pre-tRNA levels was observed in *maf1Δ* cells that were grown under restrictive conditions on YPGly (Fig. 3). Amounts of pre-tRNA-Leu, pre-tRNA-Trp and pre-tRNA-Phe were significantly decreased to various extend, from 30 % to 46 %. The modest decrease of pre-tRNA levels was observed in *maf1Δ* cells grown in YPD, but the effect of C160-NTF was not statistically significant. The observed specific effect of C160-NTF overproduction in the *maf1Δ* strain during stress conditions is consistent with the phenotypic suppression of *maf1Δ* by C160-NTF (Fig. 1A). In summary, the overdose of C160-NTF decreased tRNA transcription, thereby suppressing the defect of Maf1 inactivation that was caused by an increase in, or unbalanced levels of, various tRNAs.

3.3. Effect of C160-NTF on Pol III complex assembly

C160-NTF polypeptide is distributed in the cytoplasm and the nuclei (Fig. 1D). A possible reason for impairments in transcription activity is a direct interaction of C160-NTF polypeptide that interferes the Pol III assembly, a process which starts in the cytoplasm and is completed in the nucleus. To appreciate the physiological importance of such a regulatory event, we focused our study on effects of Maf1 and C160-NTF overproduction on interactions between individual subunits and the Pol III core. Using an HA-specific antibody, the entire Pol III complex was immunopurified from cells that expressed C160-HA, and the association between different subunits and the complex were determined by co-immunoprecipitation (co-IP). We conducted co-IP analysis using Pol III-specific available antibodies that recognize subunits which are involved in the control of Pol III initiation (C34 and C82 subunits) and elongation (C53 subunit). We examined whether their association with the Pol III core is linked to the presence of Maf1 and C160-NTF overproduction (Fig. 4). Wild type and *maf1Δ* cells, optionally carrying a plasmid that encoded C160-NTF, were grown in glucose medium at 30 °C, harvested in the exponential phase (YPD) or transferred to a medium with glycerol, and incubated at 37 °C for 2 h (YPGly). Western blot analyses of equal amounts of crude extracts showed that the transfer to repressive conditions on YPGly downregulated C160, C82 and C53 levels (Fig. 4A and B), which is consistent with our previous data (Leśniewska et al., 2019). In contrast, expression of C34 was not affected. The interaction between C160 and other subunits was estimated as the efficiency of co-IP relative to the wild type strain on YPD (Fig. 4C and D). The transfer of the control wild type strain to repressive conditions on YPGly resulted in a marked decrease in the C160-C34 association, despite no differences in the level of total extract of C34. Mutation *maf1Δ* also led to a decrease in C160-C34 association, by 65 % on YPGly, whereas the associations between C160 and C82 or C53 were decreased to a lesser extent. The observed changes in C160-C34 associations were consistent with the role of the C34 subunit in the Maf1-
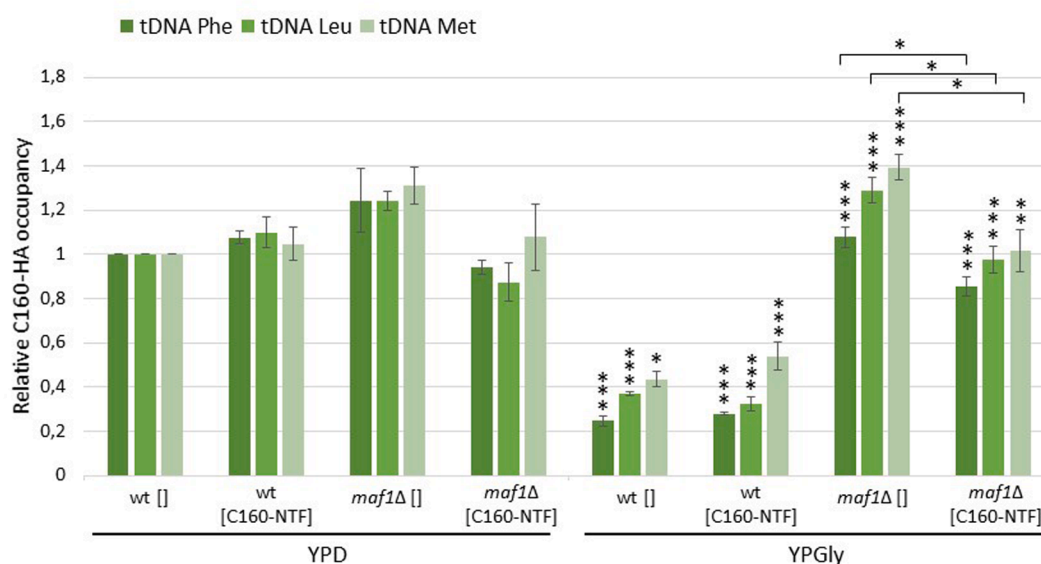
Fig. 2. Overproduction of C160-NTF downregulates Pol III occupancy in *maf1Δ* cells. The control strain (wt) and isogenic *maf1Δ* mutant strains that expressed C160 with an HA epitope and optionally overexpressing C160-NTF were grown in minimal medium overnight, then transferred to glucose medium and harvested in the exponential phase (YPD) or shifted to glycerol medium and cultivated in YPGly for 2 h at 37 °C (YPD→YPGly protocol). Cross-linked chromatin was immunoprecipitated with antibodies against HA, followed by quantitative real-time PCR with primers that were specific for tDNA-Phe, tDNA-Leu and tDNA-Met. Occupancy is represented as a percentage of immunoprecipitation over input chromatin and the wild type strain under standard conditions (YPD) was assumed to be 1. Bars represent the mean ± standard error of the mean (SEM) of three independent experiments. *p* value was calculated using a two-tailed *t*-test. The *p* values calculated for C160-HA occupancy to all tested tDNAs as ratios wt YPD/wt YPGly with or without C160-NTF, wt YPGly/*maf1Δ* YPGly with or without C160-NTF and *maf1Δ* YPGly/*maf1Δ* YPGly[C160-NTF] showed statistical significance where ***, ** and * asterisks indicate, respectively, *p* value < 0.005, < 0.01 and < 0.05.
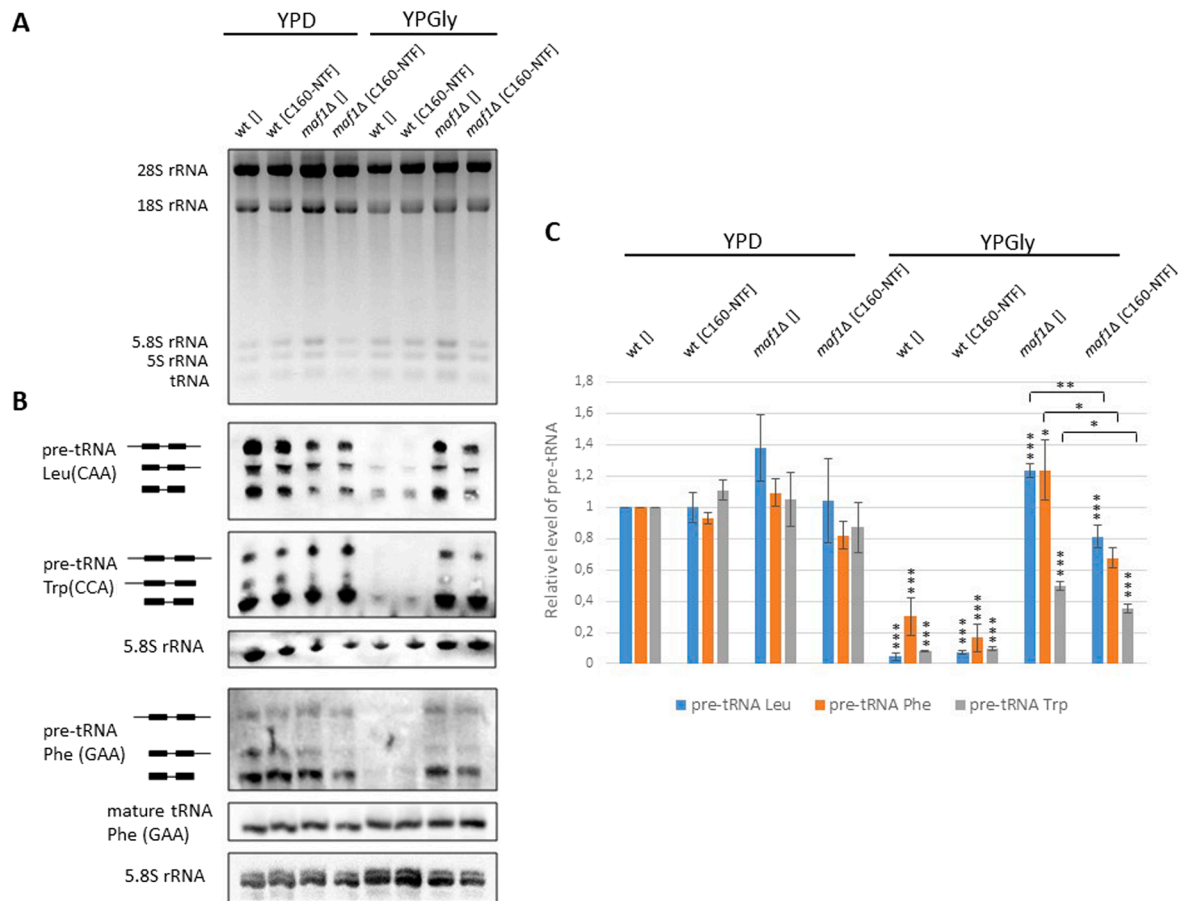
Fig. 3. Overproduction of NTF downregulates levels of tRNA precursors in *maf1Δ* cells. Strains and growth conditions were the same as in the Fig. 2 legend. Total RNAs isolated from yeast cells were analyzed on agarose gel that was stained with EtBr (A) or subjected to northern hybridization (B) with probes that were specific for intron-containing tRNA-Leu, tRNA-Trp and tRNA-Phe which detect primary transcripts (designated ▬■▬■▬), 5′ or 3′- and 5′, 3′-end-matured introns that contained tRNA precursor forms (designated ▬■■■ or ■■■▬ and ■■■, respectively). The tRNA-Phe probe also recognizes mature tRNA. 5.8S rRNA served as a loading control. Amounts of primary transcripts (▬■▬■▬) were normalized to the loading control and calculated relative to amounts in the wt strain under standard conditions (YPD), which was assumed to be 1 (C). Bars represent the mean ± standard error of the mean (SEM) of three independent experiments. *p* value was calculated using a two-tailed *t*-test. The *p* values calculated for levels of pre-tRNAs as ratios wt YPD/wt YPGly with or without C160-NTF, wt YPGly/*maf1Δ* YPGly with or without C160-NTF and *maf1Δ* YPGly/*maf1Δ* YPGly[C160-NTF] showed statistical significance where ***, ** and * asterisks indicate, respectively, p value < 0.005, < 0.01 and < 0.05.

mediated repression of Pol III transcription (Vorländer et al., 2020).

Overproduction of C160-NTF had no significant effect on the levels of Pol III subunits (Fig. 4A and B) but specifically affected their interactions in the absence of Maf1 (Fig. 4C and D). Interestingly, opposite effects of C160-NTF overproduction on the association with Pol III subunits were observed in *maf1Δ* strain under favorable *vs.* repressive condition. Overdose of C160-NTF in *maf1Δ* cells decreased more than two-fold (when compared with the wild type cells) interactions between the tested Pol III subunits under favorable condition and these effects were significantly different. The strong effect of C160-NTF overproduction was observed under repressive conditions in the *maf1Δ* mutant. The C160-C53 interaction was increased two-fold compared with the wild type strain, whereas the C160-C82 and C34 interactions appeared less affected, increased by around 40 % and 20 %, respectively.

Altogether, these findings demonstrate that the overproduction of C160-NTF affects mutual interactions between Pol III subunits supporting the effect on the assembly of the Pol III complex.

4. Discussion

Maf1 is a general and global negative regulator of Pol III transcription. Deletion of the yeast gene that encodes Maf1 leads to a temperature

growth defect on medium with a non-fermentable carbon source and to the accumulation of newly synthetized tRNA precursors (Cieśla et al., 2007). In the present study, we observed the genetic suppression of *maf1Δ* by the overproduction of C160-NTF, the N-terminal fragment of the largest Pol III subunit, C160, and explored the mechanism of suppression in a molecular study. The overproduction of C160-NTF in the yeast *maf1Δ* mutant lowered pre-tRNA levels and Pol III occupancy on tRNA genes. A possible reason for the impairment in Pol III activity is an assembly defect, indicated by results of the pull-down experiments that showed alterations of associations between subunits of the complex.

The C160-NTF polypeptide comprises the part of the C160 subunit sequence that is conserved among largest catalytic subunits of other RNA polymerases and contains zinc binding domain a. The fragment that corresponds to the a domain of C160 (aa 49–121), expressed in *E. coli* extract, is a stable protein that binds zinc in an *in vitro* assay (Werner et al., 1992). Mutations of zinc binding motifs in the a domain of C160 are lethal; one of these produced a form of Pol III that was thermosensitive and unstable *in vitro*. This instability correlated with a decrease in associations between C82, C34, and C31 subunits (Werner et al., 1992).

Here, we demonstrated that the expression of C160-NTF from a multicopy yeast plasmid resulted in an abundant polypeptide that was presumably folded in its own stable conformation. Notably, C160-NTF
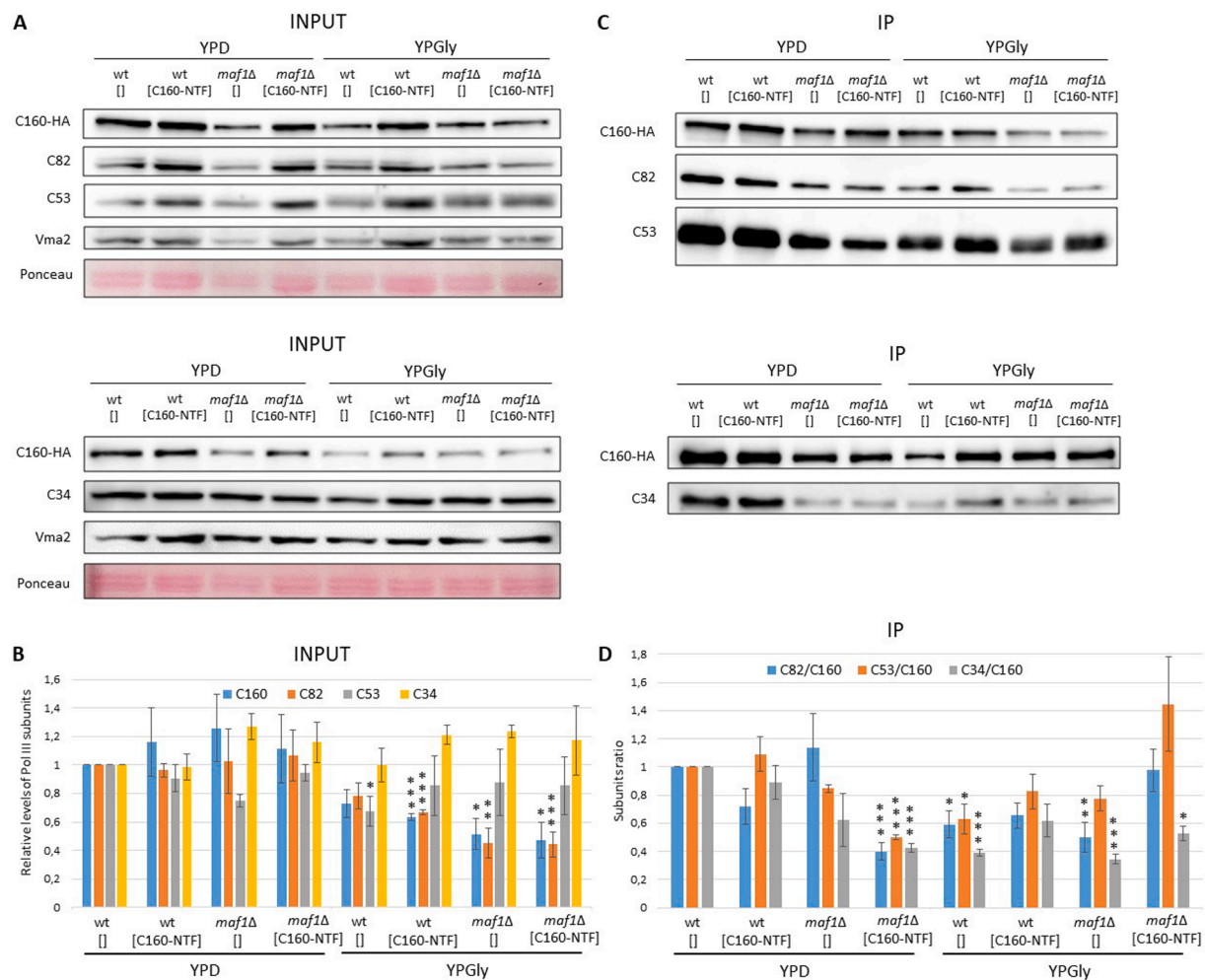
Fig. 4. Effect of C160-NTF overproduction on assembly of the Pol III complex. Extracts were prepared from isogenic wild type and *maf1*Δ mutant strains that expressed C160 with an HA epitope and optionally overexpressed C160-NTF. Cells were grown as described in the Fig. 2 legend. Pol III was immunoprecipitated from yeast extracts by HA-specific antibody. (A) Primary extracts (INPUT) and (C) IP fractions (IP) were examined by western blot with antibodies that were specific for HA, C82, C53, and C34. Loading was controlled by Vma2 protein and staining of total proteins on the blots with Ponceau red. Quantification of levels of Pol III subunits in (B) total protein extracts and (D) IP fractions. Band intensities from western blot images were quantified by ImageJ software. (B) Amounts of extracts were normalized to the loading control, Vma2 and calculated relative to amounts in the wt strain under standard condition (YPD), which was assumed to be 1. (D) The subunit ratios of IP fractions were calculated and refer to levels of respective subunits in the wild type strain under YPD condition, which was assumed to be 1. Bars represent the mean ± standard error of the mean (SEM) of three independent experiments. *p* value was calculated using a two-tailed *t*-test. The *p* values calculated for levels of Pol III subunits in INPUT as ratios wt YPD/wt or *maf1*Δ YPGly with or without C160-NTF and IP fractions as ratios wt YPD/wt YPGly, wt YPD/*maf1*Δ[C160-NTF] YPD, wt YPD/*maf1*Δ YPGly and *maf1*Δ YPGly/*maf1*Δ YPGly[C160-NTF] showed statistical significance where ***, ** and * asterisks indicate, respectively, *p* value < 0.005, < 0.01 and < 0.05.

significantly decreased levels of newly transcribed tRNA and Pol III occupancy on tRNA genes when it was overproduced in *maf1*Δ cells that were grown under repressive conditions (Figs. 2 and 3). Although C160-NTF appears to affect repression directly at the level of transcriptional units, its mode of action is obscure. Interestingly, C160-NTF had no significant effect on Pol III transcription in control wild type cells. We previously showed that C160-NTF interacted with Maf1 in an *in vitro* pull-down assay (Oficjalska-Pham et al., 2006). Therefore, C160-NTF is likely titrated by Maf1 in control cells. Moreover, a reasonable possibility is that the access of C160-NTF to Pol III regions that normally interact with Maf1 will increase in the *maf1*Δ mutant.

Maf1 achieves transcription repression through an allosteric mechanism, namely through rearrangement of the C82-C34-C31 heterotrimer, thereby locking Pol III in a conformation that is unable to initiate transcription (Vorländer et al., 2020). C82-C34-C31 is a Pol III-specific subcomplex that forms a stable heterotrimer that is positioned on the C160 clamp domain in close proximity to the Pol III stalk (Hoffmann et al., 2015).

We found that the association between the C160 subunit and C82 and C34 subunits of the heterotrimer decreased upon the overexpression of C160-NTF polypeptide (Fig. 4D, YPD panel, compare wt to wt [C160-NTF]). Interestingly, the association between C160 and C34 was lower in the *maf1*Δ mutant than in the control strain, and a further decrease was observed upon the overexpression of C160-NTF (Fig. 4D, YPD panel, compare wt and *maf1*Δ). This observation aligns with a direct interaction between Maf1 and C34 in the Pol III structure (Vorländer et al., 2020).

The association between the C160 and C53 subunits appeared to increase upon the overexpression of C160-NTF. C53 is a part of another Pol III-specific subcomplex, the C53-C37 heterodimer, that accesses the DNA-binding cleft from the opposite side than the C82-C34-C31 subcomplex (Fernández-Tornero et al., 2010; Hoffmann et al., 2015). C53 becomes phosphorylated under stress conditions (Lee et al., 2012) and is additionally modified by sumoylation (Wang et al., 2018). Notably, the C82 subunit, is also a target of sumoylation, which promotes Pol III assembly (Chymkowitch et al., 2017). These posttranslational

modifications of C53 and C82 may change local Pol III conformation, thereby increasing access of the C160-NTF polypeptide. This could lead to major rearrangements, instability of the complex, and consequently a decrease in transcription.

Interestingly, effects of C160-NTF overproduction on the association with Pol III subunits were opposite in the *maf1Δ* strain under favorable *vs.* repressive condition. Under favorable condition, an overdose of C160-NTF reduced the interactions between the tested Pol III subunits, and after transferring *maf1Δ* cells to restrictive condition, it improved the binding of Pol III subunits. We conclude that in the absence of Maf1, the C160-NTF fragment alters Pol III interactions in a carbon source-dependent manner. Pol III subunits C82, C53, and C34 may be held by the C160-NTF, which blocks the correct assembly of the Pol III complex under glucose condition. On the contrary, under repressive condition, the C160-NTF fragment is possibly incorporated to sites which are normally occupied by Maf1.

The observed changes in associations among subunits may reflect alterations of assembly of the Pol III complex. Pol III is a multisubunit complex that is assembled through a multistep process that occurs in the cytoplasm and nucleus with the aid of auxiliary factors (Turowski and Boguta, 2021). The intracellular localization of C160-NTF includes the cytoplasm and nuclei (Fig. 1D). Through direct interactions with some subunits, C160-NTF could affect the formation of Pol III subcomplexes at early steps that occur in the cytoplasm or final assembly of the active Pol III structure in the nucleus.

CRediT authorship contribution statement

Aleksandra Łopusińska: Visualization, Investigation, Validation, Formal analysis, Data curation. **Malak Farhat:** Validation, Formal analysis, Data curation. **Małgorzata Cieśla:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, 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.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2024.148839.

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