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Fluoropyrimidines trigger decay of hypomodified tRNA in yeast

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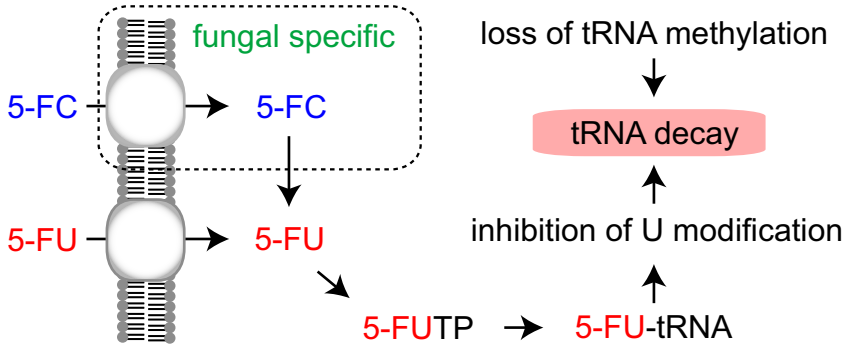
Abstract

Therapeutic fluoropyrimidines 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC) are in long use for treatment of human cancers and severe invasive fungal infections, respectively. 5-Fluorouridine triphosphate represents a bioactive metabolite of both drugs and is incorporated into target cells' RNA. Here we use the model fungus *Saccharomyces cerevisiae* to define fluorinated tRNA as a key mediator of 5-FU and 5-FC cytotoxicity when specific tRNA methylations are absent. tRNA methylation deficiency caused by loss of Trm4 and Trm8 was previously shown to trigger an RNA quality control mechanism resulting in partial destabilization of hypomodified $tRNA^{Val}_{AAC}$. We demonstrate that, following incorporation into tRNA, fluoropyrimidines strongly enhance degradation of yeast $tRNA^{Val}_{AAC}$ lacking Trm4 and Trm8 dependent methylations. At elevated temperature, such effect occurs already in absence of Trm8 alone. Genetic approaches and quantification of tRNA modification levels reveal that enhanced fluoropyrimidine cytotoxicity results from additional, drug induced uridine modification loss and activation of $tRNA^{Val}_{AAC}$ decay involving the exonuclease Xrn1. These results suggest that inhibition of tRNA methylation may be exploited to boost therapeutic efficiency of 5-FU and 5-FC.

Graphical abstract



Introduction

Transfer RNA is extensively modified and budding yeast *Saccharomyces cerevisiae* has served as an important model system to establish modification pathways and components (1,2). Modifications within the anticodon loop cooperate to maintain translational efficiency and fidelity (3–9). Modifications outside of the anticodon loop may also cooperate to stabilize tRNA and protect it from degradation by rapid tRNA decay (RTD) (10–12). RTD involves exonucleases Rat1 and Xrn1 (13) and acts on specific tRNAs lacking combinations of modifications when cells are subjected to mild heat stress (14,11). Thus, RTD causes strong negative genetic interaction of several modification genes when the corresponding double mutants are exposed to elevated temperature. Since tRNA decay is strongly triggered by elevated temperature, no negative genetic interaction between the RTD relevant modification genes is observed at regular growth temperature. One of the best characterized scenarios for RTD involves specific degradation of $tRNA^{Val}_{AAC}$ (Figure 1) when Trm8 dependent m^7G (7-methylguanosine) (15) and Trm4 dependent m^5C (5-methylcytosine) (16) are simultaneously absent. A *trm4 trm8* double mutant is viable without obvious growth defects at 30°C but completely ceases growth when shifted to 37°C. This effect is correlated with a rapid degradation of $tRNA^{Val}_{AAC}$, while other tRNAs that also lack m^5C and m^7G are not similarly destabilized (10,14). Consistently, overexpression of $tRNA^{Val}_{AAC}$ alone is sufficient to restore growth of the double mutant at the RTD inducing condition (12). Mutations in the RNA exonuclease genes *RAT1* or *XRN1* and in the bisphosphate-3′-nucleotidase gene *MET22* are known to suppress tRNA degradation and the associated growth defect (14).

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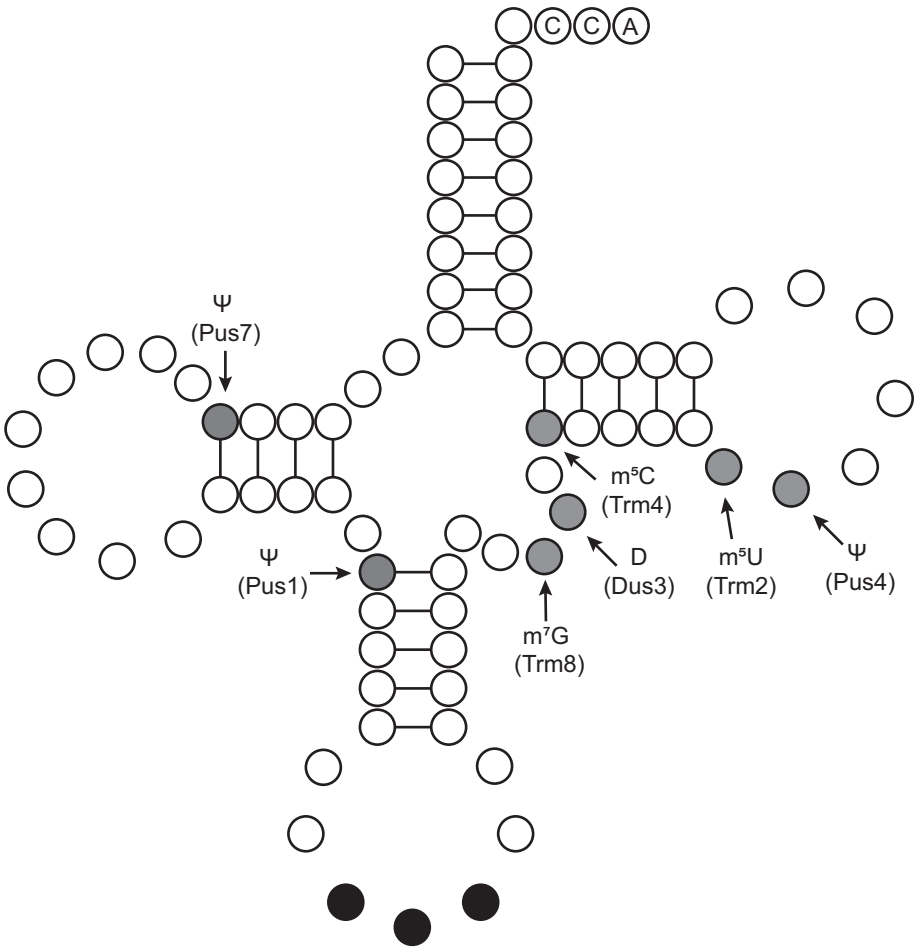

Figure 1. Sketch depicting the secondary structure of $tRNA^{Val}_{AAC}$ (43). Modifications analyzed in this paper are shown in grey circles with arrows indicating the modifying enzyme and the resulting modification. Anticodon loop is indicated in black. Ψ = pseudouridine (Pus1, Pus4, Pus7), m^5U = 5-methyluridine (Trm2), m^7G = 7-methylguanosine (Trm8), m^5C = 5-methylcytosine (Trm4). Modifications are abbreviated according to (44).

Interestingly, mutations in some tRNA modification genes with no or mild growth phenotypes result in strongly enhanced sensitivity to the cancer drug 5-fluorouracil (5-FU) when combined with moderate heat stress and this phenotype was also identified in *trm8* single mutants (17). In this paper, we demonstrate that 5-FU triggers $tRNA^{Val}_{AAC}$ destabilization in *trm8* mutants at elevated temperature and in *trm4 trm8* double mutants at regular growth temperature. The associated 5-FU sensitivity is suppressed in absence of either Met22 or Xrn1 and upon overexpression of $tRNA^{Val}_{AAC}$. We further verify that some (but not all) uridine modifications are inhibited by 5-FU and provide evidence for a toxic effect of such modification inhibition in absence of m^5C and m^7G. Since the related antifungal fluoropyrimidine 5-FC (5-fluorocytosine) has an equal effect on *trm4 trm8* mutants, the described drug induced tRNA decay might be of relevance for antifungal therapy in the future.

Materials and methods

Strains and cultivation

Yeast strains used or generated in this study are listed in Supplementary Table S1. For cultivation, yeast cells were grown in either YPD media (1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete (SC) media (0.67% yeast nitrogen base, 2% glucose) supplemented with the necessary amino acids for selection (e.g. gene deletions or plasmid transformation). 5-Fluorouracil (Sigma, F6627-1G) or 5-fluorocytosine (TCI, F0321) were added as indicated. For the negative selection of URA$^+$ strains, 0.01% 5-fluoroortic acid (5-FOA) was added to SC media (18). Gene deletions were introduced utilizing PCR based systems described in (19,20). Correct gene deletion was verified by forward/reverse primers positioned outside of the target gene and within the gene deletion cassette. Primers used for deletion and verification are listed in Supplementary Table S2. The genes *tV(AAC)* and *TRM8* were respectively cloned into YEplac195, resulting in the plasmids pRK61 and pKG24. YEplac195 served as an empty vector control. Transformation of plasmids was performed as described in (21).

RNA extraction

For bulk RNA extraction, yeast strains were pre-grown in YPD overnight and then subcultured in fresh media the next day. Cultures were grown at 30°C for approximately three hours to an OD_{600} ~1 and then shifted to 37°C or stressed with 5-FU/5-FC at 30°C/37°C for 5 h. After 5 h, the OD_{600} was measured again and cells were harvested. Following a wash step, the pellet was suspended in 500 µl NucleoZOL (Macherey-Nagel, Germany) and ~300 µl glass beads were

added. Lysis occurred through bead beating five times for 60 s intermitted by chilling the samples on ice for 5 min. Following the addition of 200 µl chloroform, the suspension was vortexed vigorously, incubated for 5 min, and centrifuged for 15 min at 15 000 rpm. The aqueous phase containing the RNA was transferred into a new reaction tube and the previous step was repeated. After another incubation and centrifugation, the clear aqueous phase was once again transferred into a new tube and precipitated with isopropanol at -20°C for 30 min. RNA was then pelleted by centrifugation for 30 min at 15 000 rpm and washed twice with 70% ethanol. Subsequently, RNA was dried at 50°C and dissolved in DEPC-treated water.

tRNA isolation and analysis of uridine modification profiles

Wild type (WT) cells were pre-grown in YPD overnight and then subcultured in fresh media the next day. After growth for approximately 3 h ($OD_{600} \sim 1$), cultures were stressed with different concentrations of 5-FU for 5 h, before the OD_{600} was measured again and cells were harvested by centrifugation. tRNA was then isolated as described in (22), hydrolyzed to nucleoside level and analyzed via liquid chromatography–tandem mass spectrometry (LC–MS) measurements as described previously (23), with slight adjustments. For modifications with high abundance (5-FU, D, ψ and m^5U) 200 ng of digested RNA spiked with 50 ng of internal standard were analyzed, while for modifications with low abundance (ncm^5U, mcm^5U, mcm^5s^2U, Um) the analyzed amount was increased to 1 µg of digested RNA sample spiked with 100 ng of internal standard. For absolute quantification of biological triplicates, internal and external calibration was applied separately for both sample sets as described in (24) with the total amount of modified nucleosides being normalized to the amount of uridines.

Northern analysis

RNA was separated on a 10% polyacrylamide gel (19:1), containing 8 M urea and run for 40 mins at 180 V. Transfer to a positively charged Nylon membrane (Roche) occurred for 45 minutes at 0.1 A and 12 V before RNA was UV-crosslinked to the membrane for 2 min on a UV table. For methylene blue staining, the membrane was equilibrated in 5% acidic acid for 10 min and then stained in 0.02% methylene blue in 0.3 M sodium acetate for 5 min. Hybridization and detection were followed as described in (25). Probes were labeled with digoxygenin and are listed in Supplementary Table S3. To determine the linear range between the sample concentration and the signal intensity during detection for the utilized probes, a concentration gradient of WT RNA samples was analyzed (Supplementary Figure S1). Northern Blots were quantified using the software Image Studio Lite 5.0. The signal intensity was normalized to the corresponding WT control. Quantifications and repeat experiments ($n = 3$) are found in Supplementary Table S4 and Supplementary Figure S6.

Results

5-FU effect in *trm8* single mutants

Since Trm8 dependent m^7G was shown (along with Trm4 dependent m^5C) to specifically protect $tRNA^{Val}_{AAC}$ from RTD (10,14), we analyzed whether an RTD variant might cause the *trm8* 5-FU phenotype. We confirm that *trm8* mutants exhibit sensitivity to 5-FU at 37°C and demonstrate robust suppression of this phenotype by mutation of *MET22* or *XRN1* (Figure 2A), pointing to an involvement of an RTD-like process. In addition, overexpression of $tRNA^{Val}_{AAC}$ significantly mitigates the 5-FU phenotype (Figure 2B), suggesting that tRNA decay of $tRNA^{Val}_{AAC}$ might occur during drug exposure. To directly test this, we compared $tRNA^{Val}_{AAC}$ levels in WT, *trm8* and *trm8 met22* mutants using Northern analysis (Figure 2C). At 30°C in absence of 5-FU, no major differences between the $tRNA^{Val}_{AAC}$ levels in the different strains were observed, whereas a 5 h shift to 37°C resulted in a 50% reduction of the $tRNA^{Val}_{AAC}$ levels in the *trm8* mutant (Supplementary Table S4), but not in the wild type or *trm8 met22* double mutant. In the presence of 5-FU (10 µg/ml) at 37°C, the $tRNA^{Val}_{AAC}$ signal of the *trm8* mutant was reduced to 10% and this effect was suppressed in *trm8 met22*. While $tRNA^{Val}_{AAC}$ levels were drastically affected by 5-FU at 37°C, levels of $tRNA^{Gly}_{GCC}$, bulk tRNA, or the small ribosomal RNAs remained nearly constant (Figure 2C). A comparable suppression of $tRNA^{Val}_{AAC}$ degradation can be observed in *trm8 xrn1* (Supplementary Figure S2A). At 30°C, the same dose of 5-FU does not affect $tRNA^{Val}_{AAC}$ levels in the *trm8* mutant (Supplementary Figure S2B). In 5-FU exposed wild type cells, $tRNA^{Val}_{AAC}$ signals routinely increased (Supplementary Table S4), suggesting some stabilizing effect of the drug when no other modification defects are present.

5-FU effect in *trm4 trm8* double mutants

Since $tRNA^{Val}_{AAC}$ is known to be destabilized by heat stress in *trm4 trm8* mutants (10,14), we investigated whether *trm4* has a similar 5-FU phenotype as observed for *trm8* and whether the two tRNA methyltransferase genes interact genetically on 5-FU containing media. While *trm4* mutants do not exhibit severe growth defects in presence of 5-FU at either temperature (Figure 3A), the *trm4 trm8* double mutant shows a severe growth defect at 5 and 10 µg/ml 5-FU in absence of heat stress (30°C). As expected, a similar growth inhibition of the double mutant also occurs at 37°C in the absence of the drug (10). Hence, strong negative interaction of *TRM4* and *TRM8* occur at 37°C but not at 30°C in absence of 5-FU and at 30°C in presence of the drug. The *trm4 trm8* double mutant 5-FU phenotype at 30°C is suppressed upon inactivation of the RTD factor Met22 or Xrn1 (Figure 3A). Comparable to the *trm8* single mutant, the double mutant 5-FU phenotype is also suppressed by overexpression of $tRNA^{Val}_{AAC}$ (Supplementary Figure S3A). Quantification of $tRNA^{Val}_{AAC}$ levels in wild type, *trm4 trm8* and *trm4 trm8 met22* mutants revealed a substantial decline to ∼50% of wild type level in the double mutants already at 30°C and a more severe loss to ∼10% at either 37°C or upon 5-FU addition at 30°C (Figure 3B). In contrast to the *trm8* single mutant, no destabilization of $tRNA^{Val}_{AAC}$ was observed in a *trm4* single mutant exposed to 5-FU at 37°C (Supplementary Figure S2C). Hence, absence of Trm4 dependent m^5C sensitizes $tRNA^{Val}_{AAC}$ for 5-FU induced decay only when m^7G is absent as well. As expected for an RTD-like effect, levels of $tRNA^{Val}_{AAC}$ are restored in all conditions in the *trm4 trm8 met22* triple mutant. Mutation of *XRN1* similarly suppresses 5-FU induced tRNA degradation and 5-FU sensitivity in the *trm4 trm8* strain (Supplementary Figure S3B).

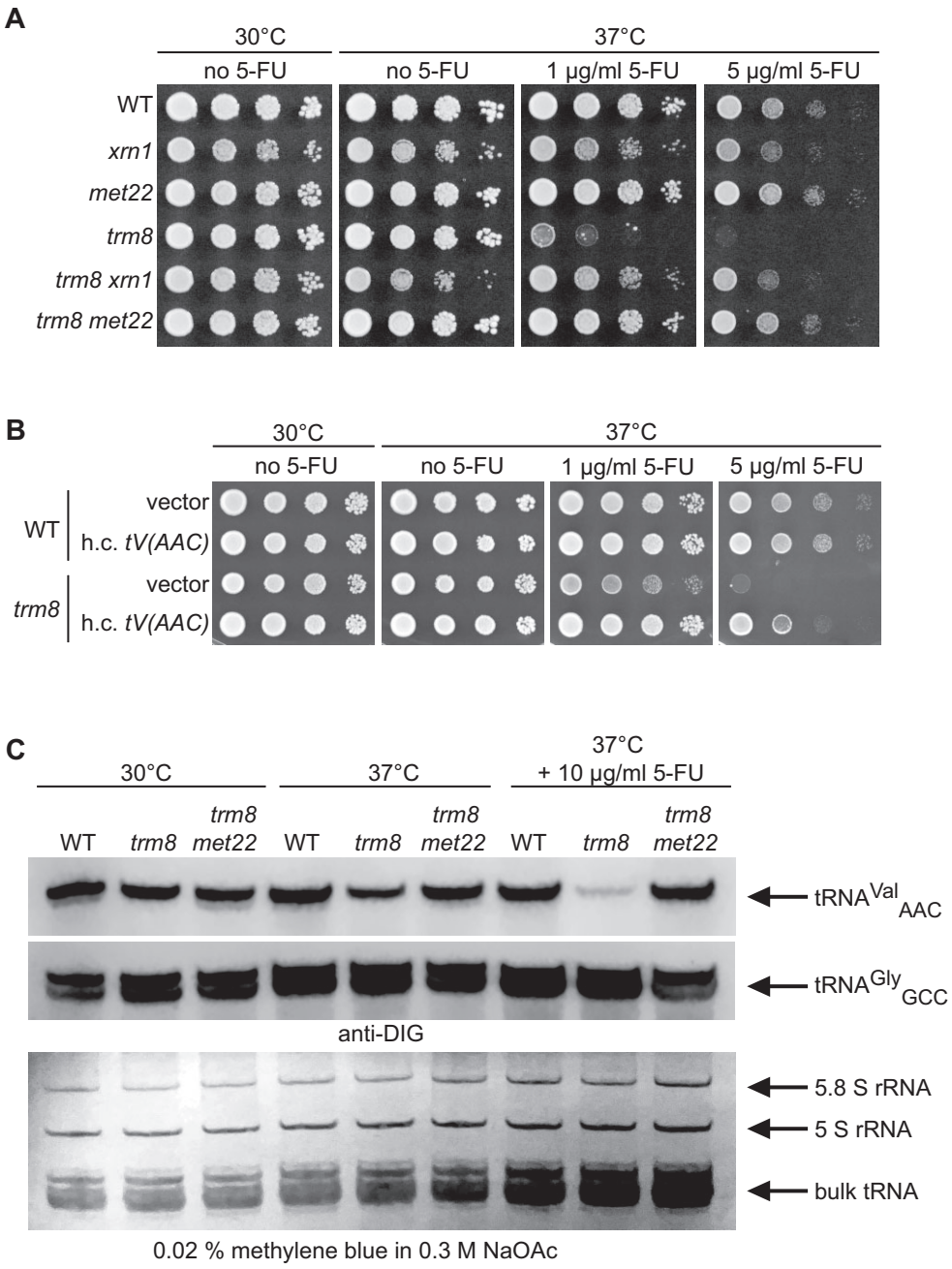

Figure 2. tRNA levels and 5-FU response of yeast strains lacking *TRM8*. (**A**) Phenotypic growth assay of indicated strains. Serial dilutions of cells were plated on YPD containing indicated concentrations of 5-FU and incubated for two days at either 30°C or 37°C. (**B**) Changes in 5-FU sensitivity upon overexpression of $tRNA^{Val}_{AAC}$. Indicated strains carry either an overexpression construct for $tRNA^{Val}_{AAC}$ (h.c. *tV(AAC)*) or the empty vector control (vector) and were assayed as described in (A). (**C**) Levels of $tRNA^{Val}_{AAC,}$ $tRNA^{Gly}_{GCC}$ and 5S/5.8 S rRNA after temperature stress (37°C) alone or with additional 5-FU exposure (37°C + 10 µg/ml 5-FU). Cells were grown to early exponential phase at 30°C and shifted to indicated conditions for 5h. Total RNA was used for Northern analysis with probes for either $tRNA^{Val}_{AAC}$ or $tRNA^{Gly}_{GCC}$. The methylene blue stained membrane was used to monitor 5.8S and 5S rRNA levels as well as bulk tRNA amounts.

5-FU effect on other double tRNA modification mutants

In addition to *trm4*, other tRNA modification defects introduced in a *trm8* mutant background were also shown to result in the activation of heat induced RTD (10). For example, double mutants *trm8 dus3* (lacking m^7G and D_{47}) and *trm8 pus7* (lacking m^7G and Ψ_{13}) exhibit a synthetic temperature sensitive (TS) phenotype which is linked to $tRNA^{Val}_{AAC}$ degradation (10). To test for similarities between RTD and fluoropyrimidine phenotypes in tRNA modification mutants, we also analyzed *trm8 dus3* and *trm8 pus7* double mutants for their response to 5-FU. As for the *trm4 trm8* mutant, both of the additional double mutants exhibit a severe 5-FU sensitivity at 30°C, which is suppressed by mutation of *MET22* (Supplementary Figure S4). Exemplified for the *trm8 pus7* mutant, this phenotype again correlates with a drug induced, *MET22* dependent reduction of $tRNA^{Val}_{AAC}$ levels (Supplementary Figure S4B). Hence, other modification defect

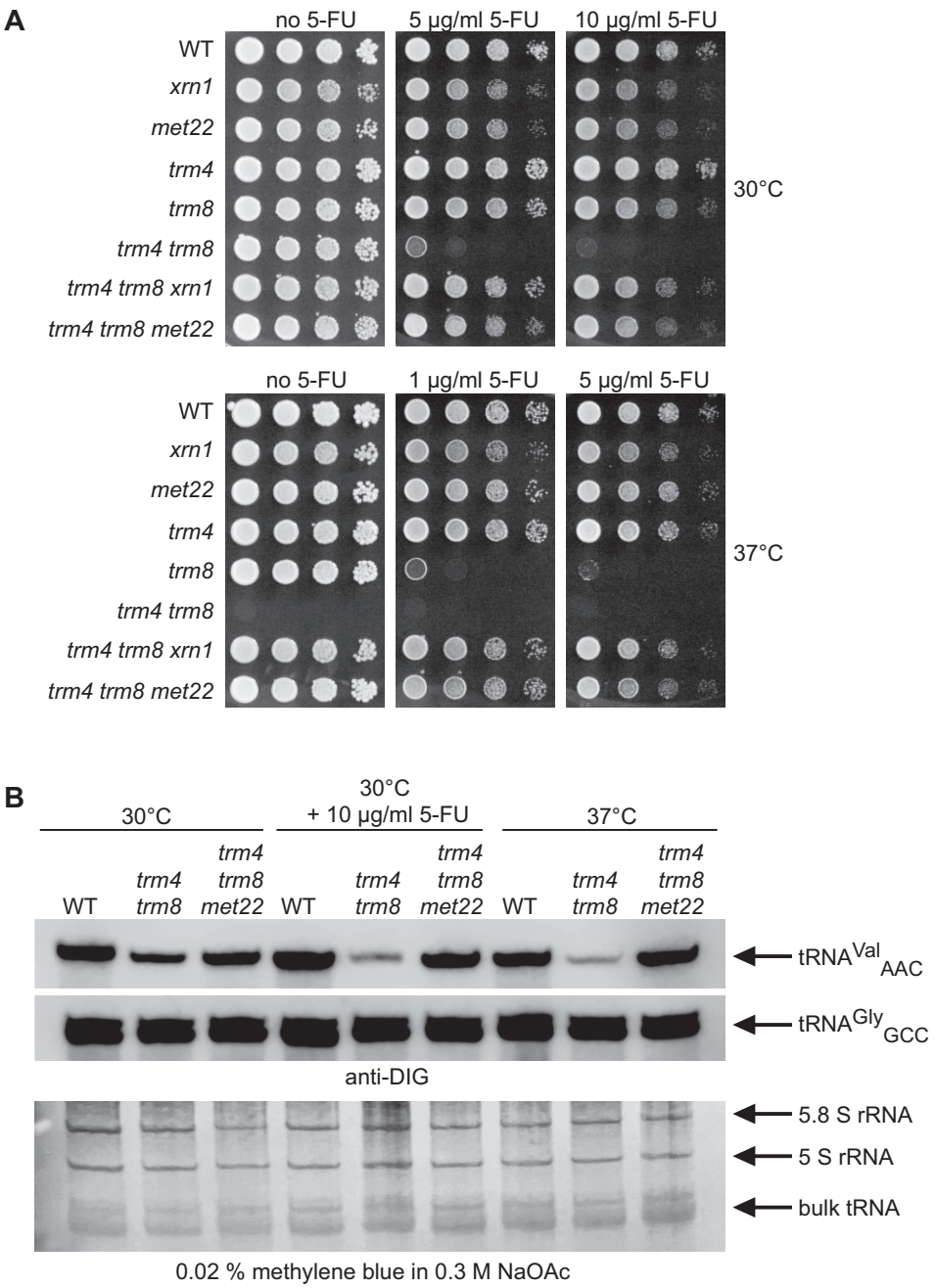

Figure 3. tRNA levels and 5-FU response of yeast strains lacking *TRM4* and *TRM8*. (**A**) Phenotypic growth assay of indicated strains on 5-FU containing and drug free medium at 30°C or 37°C incubation temperature. The assay was prepared as described in Figure 2. (**B**) Levels of $tRNA^{Val}_{AAC}$, $tRNA^{Gly}_{GCC}$ and 5S/5.8 S rRNA after temperature stress (37°C) or after 5-FU exposure in absence of temperature stress (30°C + 10 µg/ml 5-FU). Cultures were exposed to the indicated condition as described in Figure 2. RNA species were detected by Northern Blot analysis or direct staining of the membrane as described in Figure 2.

combinations known to sensitize $tRNA^{Val}_{AAC}$ for RTD also sensitize for drug induced tRNA decay.

5-FC stress results in comparable effects in double mutant *trm4 trm8*

5-Fluorocytosine (5-FC) is another therapeutic fluoropyrimidine used for treatment of systematic fungal infections (26,27). The drug's uptake is mediated by the cytosine/adenine permease Fcy2 as well as five different homologs (26,28) before the fungal specific deaminase Fcy1 converts 5-FC to 5-FU (29). Consequently, 5-FC stress in *trm4 trm8* should result in degradation of $tRNA^{Val}_{AAC}$ as well. To test this hypothesis, we compared the effects of 5-FU and 5-FC on *trm4 trm8*. As expected, the double mutant shows a severe growth defect during stress with both fluoropyrimidines (5 µg/ml) at 30°C (Supplementary Figure S5A) as well as a degradation of $tRNA^{Val}_{AAC}$ (Supplementary Figure S5B). To confirm the necessary step of deamination of 5-FC to 5-FU through Fcy1, we created the triple mutant *trm4 trm8 fcy1*. Without the deaminase Fcy1, the 5-FC growth phenotype is suppressed in the triple mutant

(Supplementary Figure S5A) and tRNA$^{Val}_{AAC}$ is no longer degraded during 5-FC stress (Supplementary Figure S5B). Therefore, both fluoropyrimidines seem to trigger tRNA decay in a similar manner.

Time course of drug induced tRNA decay

Time course analysis of RTD in the *trm4 trm8* mutant revealed that tRNA decay occurs very rapidly after temperature shift, as tRNA$^{Val}_{AAC}$ levels declined by ∼50% within 30 min at 37°C (10). To test whether further mechanistic similarities between RTD and 5-FU induced tRNA decay exist, we conducted a time course analysis comparing tRNA$^{Val}_{AAC}$ levels in wild type and *trm4 trm8* double mutants after shift to 5-FU containing media and to 37°C. Consistent with a previous study (10), tRNA$^{Val}_{AAC}$ levels decline strongly after 30 minutes of heat stress (Figure 4G, H). The 5-FU effect was observable in the double mutant after 180 min but not at earlier time points (Figure 4C, D). 240 min of 5-FU exposure were required to drop tRNA$^{Val}_{AAC}$ levels below 50%. As observed before (Figure 3), 5-FU and mild heat stress affected tRNA levels only in the double mutant, but not in the wild type (Figure 4A, B, E, F). These results reveal a clear difference between heat induced and drug induced tRNA decay—while the former is known to occur within minutes after heat stress (10), the fluoropyrimidine effect occurs with a significant delay (>180 min). The difference between heat induced RTD and drug induced tRNA decay in terms of speed suggests mechanistic differences.

5-FU induced changes in tRNA modification

5-FU is known to be taken up into fungal target cells by specific permeases and is subsequently metabolized to 5-FUTP, which is used by RNA polymerases instead of UTP (30,31). Hence, fluorinated uridine is detectable in cellular RNA species after 5-FU exposure and was shown to result in inhibition of different uridine specific modifications (32). It is unknown, however, whether all uridine modifications are inhibited. Multiple wobble uridine modifications (xcm^{5}U), which are dependent on the Elongator complex as well as 2′ O-methyluridine (Um) were not covered in previous studies (33). To reveal which uridine modifications are affected under the drug exposure conditions applied in this study, we profiled modifications in total tRNA after 5-FU exposure by LC-MS. As expected, increasing doses of 5-FU in the media resulted in growth inhibition (Figure 5A) and an increased detection of 5-FU in total tRNA preparations (Figure 5B). For the uridine specific modifications, we observed a significant reduction of m^{5}U and Ψ, whereas D, Um, ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U were not affected (Figure 5C, D). 10 μg/ml of 5-FU was sufficient to achieve ∼30% reduction of m^{5}U and Ψ, whereas higher doses were hardly more efficient. Notably, strains stressed with 5-FU showed reduced growth (∼1 cell division during 5h drug exposure) compared to untreated cells (∼2 cell divisions). To correlate our LC-MS results to the decay of tRNA$^{Val}_{AAC}$ in *trm4 trm8*, we increased the dose of 5-FU to 20 and 50 μg/ml to match the conditions of the LC–MS measurement. As shown in Figure 5E, increasing the concentration of 5-FU from 10 to either 20 or 50 μg/ml does not lead to a further destabilization of tRNA$^{Val}_{AAC}$ levels in *trm4 trm8*.

Lethal effect of additional modification defects in *trm4 trm8*

Since under conditions of 5-FU exposure, mainly Ψ and m^{5}U were affected, we tested how individual deletions of genes encoding specific Ψ synthases or the m^{5}U introducing methyltransferase Trm2 affected viability of a *trm4 trm8* double mutant. If the severe growth defect on 5-FU containing media is due to the drug induced loss of individual uridine modifications, a gene deletion disabling the specific uridine (U) modifier could have deleterious effects. To test this experimentally, we first transformed the healthy *trm4 trm8* double mutant with a counter selectable *URA3-TRM8* plasmid. Next, *PUS1*, *PUS4, PUS7* and *TRM2* (encoding specific pseudouridine synthases and the m^{5}U forming methyltransferase, respectively) were individually deleted. Afterwards, growth on media containing 5-FOA, which only permits growth of cells having lost the *URA3-TRM8* plasmid was monitored. Each of the U-modifiers studied has a target in tRNA$^{Val}_{AAC}$. As shown in Figure 6A, *trm4 trm8* double mutants are viable on 5-FOA, but none of the triple mutants lacking each additionally one specific U-modifier gene. Thus, each of the Ψ synthases (Pus1, Pus4 and Pus7) and the m^{5}U specific methyltransferase Trm2 are required for viability in absence of *TRM4* and *TRM8*. Therefore, chemical inhibition of m^{5}U and Ψ formation by 5-FU could mechanistically explain the severe drug sensitivity of the *trm4 trm8* double mutant, as it partially mimics the modification status of the lethal triple mutant combinations studied. To test whether the inviability of the tested triple mutants could be due to an RTD-like effect, we additionally deleted the RTD factor *MET22* and tested for changes in growth on 5-FOA media. As shown in Figure 6A, *met22* mutation indeed permitted growth on 5-FOA and therefore suppresses the inviability of the analyzed triple mutants. This result suggests a further decreased tRNA stability via activation of decay in the triple mutants as compared to *trm4 trm8*. To check whether the inviability of the triple modification mutants could be linked to tRNA$^{Val}_{AAC}$ destabilization, we exemplarily tested whether elevated levels of this tRNA could suppress inviability of one of the combinations. As shown in Figure 6B, viability of the *trm4 trm8 trm2* triple mutant is indeed restored in presence of the tRNA$^{Val}_{AAC}$ overexpression construct, suggesting that lethal effects are due to reduced levels of this tRNA.

Discussion

In yeast and human cells, 5-FU can be metabolized to 5-FUTP and subsequently incorporated into different RNA species (31,34). A consequence of such 5-FUTP misincorporation is the inhibition of modification enzymes targeting uridines (35–38).

It was speculated that 5-FU sensitive phenotypes of different yeast mutants lacking tRNA modification enzymes might be due to additional modification loss and subsequent tRNA destabilization (17). In our study, we aimed to define whether (i) 5-FU induced tRNA decay occurs in yeast, (ii) reveal target tRNAs and (iii) identify potentially involved pathways. Of note, combined absence of specific tRNA modifications was shown to result in a selective destabilization of hypomodified tRNAs via activation of exonucleases Rat1 and Xrn1 in a process termed rapid tRNA decay (RTD) (10,14). Hence, we

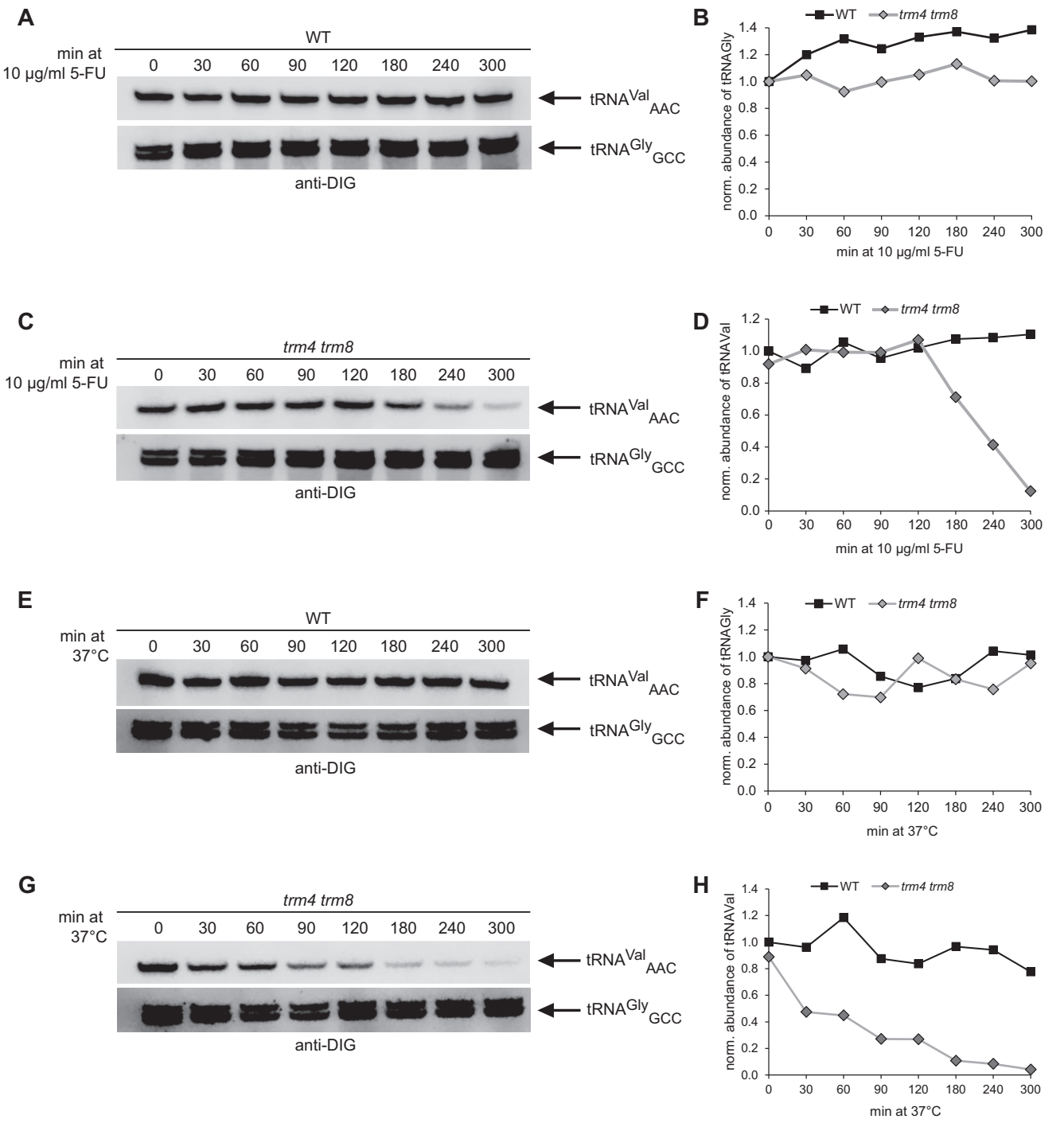

Figure 4. Time course of $tRNA^{Val}_{AAC}$ degradation in WT and *trm4 trm8*. (**A, C, E, G**) Cells were grown to an $OD_{600} = 1$ and then either stressed with 10 µg/ml 5-FU at 30°C or shifted to 37°C for the indicated times. RNA was isolated and used for Northern blot analysis with probes for either $tRNA^{Val}_{AAC}$ or $tRNA^{Gly}_{GCC}$. (**B, D, F, H**) Comparison of normalized abundance of either $tRNA^{Gly}_{GCC}$ or $tRNA^{Val}_{AAC}$ in WT cells and *trm4 trm8*.

speculated that 5-FU induced tRNA decay might be mechanistically related to the well described RTD pathway.

We tested whether 5-FU phenotypes of tRNA modification mutants are affected by loss of the RTD exonuclease Xrn1 or Met22, a bisphosphate-3′-nucleotidase preventing the accumulation of an Xrn1/Rat1 inhibitory metabolite (39,14). As exemplified for *trm8* mutants lacking m^7G, we show that the mutants' 5-FU phenotype depends on the presence of both Xrn1 and Met22, suggesting an RTD-like mechanism. Since combined absence of Trm8 dependent m^7G and Trm4 dependent m^5C triggers selective decay of $tRNA^{Val}_{AAC}$ upon heat stress (10,14), we tested whether this tRNA species might also be targeted by 5-FU induced decay.

Indeed, *trm8* mutants exhibit severely reduced levels of this tRNA when 5-FU is applied at elevated temperatures and this effect requires Met22 and Xrn1. Since overexpression of $tRNA^{Val}_{AAC}$ is sufficient to suppress the mutants' 5-FU phenotype, it is caused by changes in abundance of just this tRNA. While this work was in progress, another study demonstrated

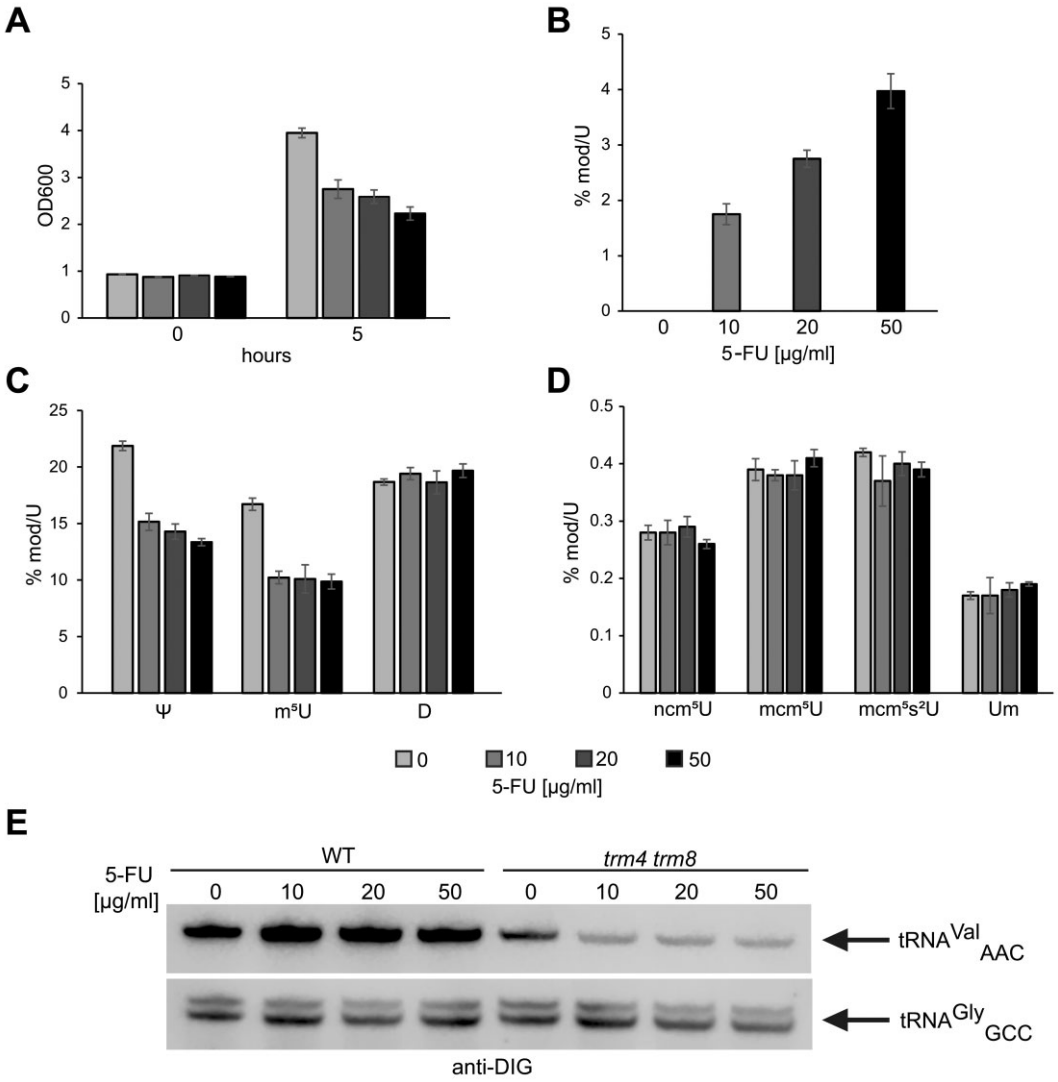

Figure 5. Profiling of uridine modifications in WT cells after 5-FU exposure. WT cells were grown to an $OD_{600} = 1$ in liquid cultures and then stressed for 5 h at 30°C with different concentrations of 5-FU. tRNA was isolated, hydrolyzed to nucleoside level and analyzed via LC–MS measurements. The total amount of modified nucleosides was normalized to the amount of uridines (% mod/U). (**A**) Cell density measurements of cultures stressed with 5-FU after 5 hours. Time point 0 marks the cell density before 5-FU stress. (**B**) Detection of 5-FU in isolated tRNA after exposure of cells to indicated concentrations of 5-FU. (**C, D**) The abundance of pseudouridine (Ψ) and 5-methyluridine (m^5U) decrease upon exposure of cells to 5-FU while levels of dihydrouridine (D), 5-carbamoylmethyluridine (ncm^5U), 5-methoxycarbonylmethyluridine (mcm^5U), 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) and 2′-O-methyluridine (Um) are not affected ($n = 3$). (**E**) Comparison of $tRNA^{Val}_{AAC}$ or $tRNA^{Gly}_{GCC}$ levels in WT and *trm4 trm8* after 5 h stress at different 5-FU concentrations.

that $tRNA^{Trp}_{CCA}$ is similarly destabilized during 5-FU exposure when Trm10 dependent m^1G is absent, revealing that fluoropyrimidine induced tRNA decay is not limited to $tRNA^{Val}_{AAC}$ (40). This suggests that different methylations are important for the stability of different tRNAs when uridine modifications are chemically inhibited (Trm4/Trm8 for $tRNA^{Val}_{AAC}$, Trm10 for $tRNA^{Trp}_{CCA}$). In addition, 5-FU incorporation was also found to inhibit pre $tRNA^{Trp}_{CCA}$ end processing and splicing, even in presence of Trm10 dependent m^1G (40). A negative effect of incorporated 5-FU on tRNA turnover might also account for our repeatedly observed increased tRNA signals in drug exposed wild type cells (Supplementary Table S4).

Destabilization of $tRNA^{Val}_{AAC}$ in *trm4 trm8* yeast mutants is strongly induced by mild heat stress, explaining a severe growth defect of the double mutant at elevated temperature, but not in absence of temperature stress (10). We show in here that combined absence of *TRM4* and *TRM8* enables 5-FU induced decay of $tRNA^{Val}_{AAC}$ already at regular temperature, resulting in a 5-FU sensitive phenotype in absence of temperature stress. In contrast, tRNA decay in *trm8* single mutant by 5-FU required temperature stress and a 5-FU phenotype is present only at elevated temperature. Since $tRNA^{Val}_{AAC}$ levels are already moderately reduced in *trm4 trm8* mutants at 30°C, temperature shift and drug exposure both activate a further decline in $tRNA^{Val}_{AAC}$ levels. Knockdown of human *TRM4* and *TRM8* orthologs in HeLa cells also increases 5-FU sensitivity, and drug exposure decreased levels of $tRNA^{Val}_{AAC}$ (41) indicating fluoropyrimidine induced tRNA decay may be evolutionary conserved. However, in human cells, heat stress did not enhance $tRNA^{Val}_{AAC}$ degradation and exonucleases potentially involved in drug induced tRNA decay remain to be identified.

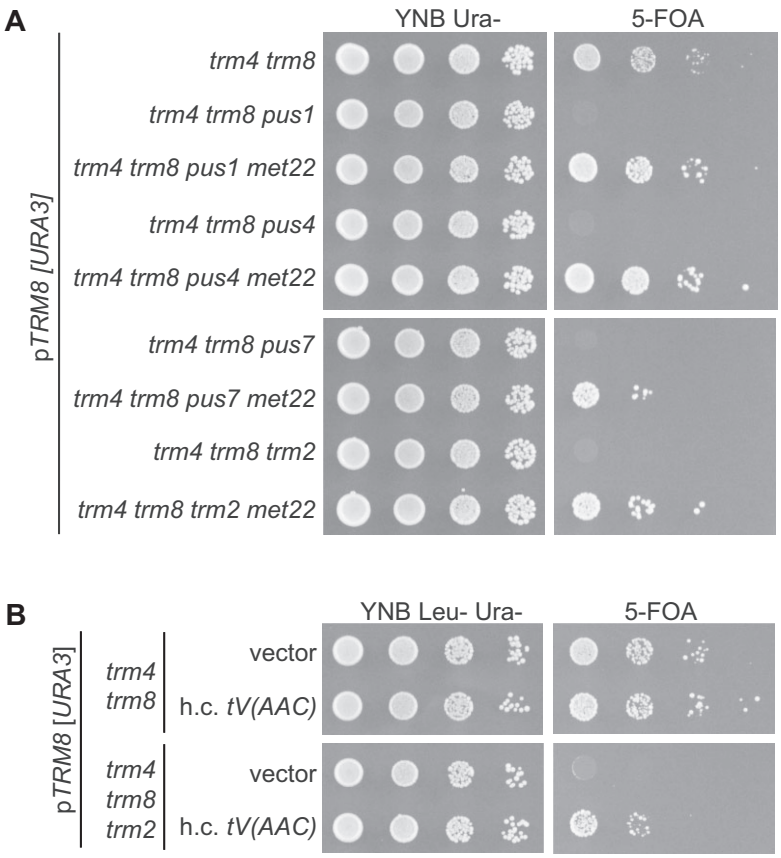

Figure 6. Effects of additional third tRNA modification gene deletion in *trm4 trm8*. The double mutant was transformed with a *URA3-TRM8* (p*TRM8*) plasmid before additional deletion of a third tRNA modification gene (*PUS1, PUS4, PUS7, TRM2*). Before spotting the indicated strains on 5-FOA at 30°C, which is lethal for cells harboring the *URA3* gene, strains were grown on YPD plates overnight. (**A**) In each triple mutant carrying p*TRM8, MET22* was additionally deleted. Absence of growth on 5-FOA medium reveals lethal mutant combinations. (**B**) Triple mutant *trm4 trm8 trm2* was transformed with either an empty vector or a high copy (h.c.) plasmid carrying *tV(AAC). trm4 trm8* serves as a control. Overexpression of *tV(AAC)* in *trm4 trm8 trm2* leads to rescue of lethality.

Drug induced tRNA destabilization is also observed in a distinct yeast double mutant (*trm8 pus7*) exhibiting lowered levels of $tRNA^{Val}_{AAC}$ and suggests pre-destabilization of this tRNA sensitizes for further decay induced by 5-FU. As the drug was shown to act as a potent inhibitor of several uridine modifications (32), this effect might be responsible for tRNA destabilization. While $tRNA^{Val}_{AAC}$ lacking the 5-FU sensitive modifications alone or lacking only the Trm4 and Trm8 dependent modifications remains at least partially stable, a combination of both effects (5-FU exposure of the *trm4 trm8* mutant) could explain the severe loss of tRNA stability as multiple modifications are affected. Since *met22* and *xrn1* mutation robustly rescues single and double mutant 5-FU phenotypes and suppresses drug induced $tRNA^{Val}_{AAC}$ decay, the negative growth phenotypes are largely caused by tRNA decay.

To compare the effect of temperature shift and 5-FU exposure on $tRNA^{Val}_{AAC}$ levels in presence and absence of Trm4 and Trm8 dependent modifications, we analyzed the time range required for tRNA levels to be affected. While heat shift of *trm4 trm8* mutants led to a decline of $tRNA^{Val}_{AAC}$ levels within minutes (10), the 5-FU effect was substantially delayed. We assume that such delay occurs because the drug affects uridine modifications only after cellular uptake and misincorporation into newly transcribed tRNA.

To verify inhibition of uridine modifications by 5-FU and to define affected and non-affected modifications, we used LC-MS analysis of tRNA samples from yeast cells treated with different concentrations of 5-FU for the same time period used in tRNA decay assays before. 5-FU exposure was previously shown to reduce the abundance of some tRNA modifications (32). However, this decrease cannot be solely attributed to 5-FUTP incorporation into tRNA as it occurs at a much higher stoichiometry. Some uridine modifiers, such as the methyltransferase Trm2, were shown to be inhibited during 5-FU stress (32,35,36,38,37). The formation of covalent tRNA-Trm2 complexes were observed upon attempted methylation at 5-FU sites, resulting in irreversible Trm2 inactivation (35). While some uridine modifications are affected by 5-FU (32), no information was available for potential effects on Um and the xcm^5U modification family.

We detected a dose dependent increase of 5-FU in total tRNA and a concomitant loss of ψ and m^5U, whereas D, Um, mcm^5U, ncm^5U and mcm^5s^2U were not affected under the conditions applied in this study. Interestingly, the smallest dose of 5-FU (10 μg/ml) reduced the detected m^5U and ψ levels by ∼30%, while higher doses were not proportionally more effective. We assume the absence of a dose dependent decrease in m^5U and ψ levels is due to stable, modified tRNA present in cells before drug exposure. Such tRNA would not be affected by 5-FU, as the drug would only inhibit new modifications

without removing those that were already present when the drug was applied. It is reasonable to assume that the lowest dose of 5-FU is sufficient to inhibit specific uridine modifiers. Higher doses of 5-FU would then still result in increased incorporation into new tRNA transcripts without further affecting modification levels, since ∼60% of modification abundance quantified are due to those tRNAs that were fully modified and already present before drug exposure. Consistently, cell densities of the 5-FU containing cultures have only doubled once during the 5h incubation time and higher doses of 5-FU (20 and 50 μg/ml) were not proportionally more effective in triggering degradation of $tRNA^{Val}_{AAC}$ (Figure 5E).

The differential response of specific uridine modifications to 5-FU might also indicate differences in reaction mechanisms. For example, while the xcm^5U modifications were unaffected, the m^5U specific Trm2 is inhibited by 5-FU because a covalent reaction intermediate with incorporated 5-FU is formed that cannot be resolved and thus results in irreversible enzyme inhibition (35). The absence of effects of 5-FU on the xcm^5U modifications might therefore hint to different catalytic reaction mechanisms between Elongator and Trm2.

To test whether growth inhibition of *trm4 trm8* mutants by 5-FU could be due to chemical inhibition of ψ and m^5U formation, we analyzed triple genetic interactions of *TRM4* and *TRM8* with individual pseudouridine synthase genes and the methyltransferase responsible for m^5U formation. If the inability of the *trm4 trm8* double mutant to grow in presence of 5-FU is due to chemical inhibition of ψ and/or m^5U, deletion of the tRNA modifier genes targeting uridines in $tRNA^{Val}_{AAC}$ might be deleterious. Indeed, each of the tested pseudouridine synthases as well as the Trm2 methyltransferase become essential in the *trm4 trm8* background. This result identifies scarce biological functions for conserved m^5U_{54} and $ψ_{55}$ and suggests that removal of additional uridine modifications in $tRNA^{Val}_{AAC}$ lacking m^5C and m^7G leads to further destabilization, likely involving RTD involved exonucleases. In support, synthetic lethal effects in the studied triple mutants are suppressed by additional mutation of *MET22* and overexpression of $tRNA^{Val}_{AAC}$ in *trm4 trm8 trm2* also rescues the lethality. Likely, accumulation of multiple modification defects in $tRNA^{Val}_{AAC}$ gradually destabilizes it by activating exonucleolytic decay and lowering its level below a certain threshold results in a complete growth arrest. In $tRNA^{Val}_{AAC}$ lacking m^5C and m^7G, this threshold is undercut when either individual U-modifiers are lost completely by mutation or by 5-FU induced inhibition of specific uridine modifications.

Since the findings reported in this study are potentially relevant for therapeutic applications of fluoropyrimidines, we also extended our investigations to the 5-FU related 5-FC, which is used in antifungal therapy (27). Because 5-FC is metabolized by the fungal specific deaminase Fcy1 to 5-FU (26,27), equivalent effects of 5-FU and 5-FC on yeast cells proficient in 5-FU deamination were expected. Indeed, we detected not only cross sensitivity of tRNA modification mutants to both drugs but also $tRNA^{Val}_{AAC}$ is destabilized by 5-FC in a Fcy1 dependent manner. Thus, inhibition of two tRNA methyltransferases (Trm4 and Trm8) greatly increases the 5-FC sensitivity and tRNA decay in the fungal model organism *S. cerevisiae*. Since RTD was demonstrated to be evolutionary conserved in phylogenetically distinct yeast species (*Schizzosacharomyces pombe*) (42), we assume that RTD and possibly drug induced tRNA decay would likely also occur in more closely related fungal pathogens such as *Candia albicans*. If so, therapeutic efficiency of 5-FC could be boosted when tRNA methyltransferase inhibitor drugs would be combined with 5-FC.

Data availability

All data are incorporated into the article and its online Supplementary material.

Supplementary data

Supplementary Data are available at NAR Online.

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Conflict of interest statement

M.H. is a consultant for Moderna Inc.

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