Hypomodified tRNA in evolutionarily distant yeasts can trigger rapid tRNA decay to activate the general amino acid control response, but with different consequences

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Abstract

All tRNAs are extensively modified, and modification deficiency often results in growth defects in the budding yeast *Saccharomyces cerevisiae* and neurological or other disorders in humans. In *S. cerevisiae*, lack of any of several tRNA body modifications results in rapid tRNA decay (RTD) of certain mature tRNAs by the 5’-3’ exonucleases Rat1 and Xrn1. As tRNA quality control decay mechanisms are not extensively studied in other eukaryotes, we studied *trm8Δ* mutants in the evolutionarily distant fission yeast *Schizosaccharomyces pombe*, which lack 7-methylguanosine at G₄₆ (m⁷G₄₆) of their tRNAs. We report here that *S. pombe trm8Δ* mutants are temperature sensitive primarily due to decay of tRNA^{Tyr(GUA)} and that spontaneous mutations in the *RAT1* ortholog *dhp1*+ restored temperature resistance and prevented tRNA decay, demonstrating conservation of the RTD pathway. We also report for the first time evidence linking the RTD and the general amino acid control (GAAC) pathways, which we show in both *S. pombe* and *S. cerevisiae*. In *S. pombe trm8Δ* mutants, spontaneous GAAC mutations restored temperature resistance and tRNA levels, and the *trm8Δ* temperature sensitivity was precisely linked to GAAC activation due to tRNA^{Tyr(GUA)} decay. Similarly, in the well-studied *S. cerevisiae trm8Δ trm4Δ* RTD mutant, temperature sensitivity was closely linked to GAAC activation due to tRNA^{Val(AAC)} decay; however, in *S. cerevisiae*, GAAC mutations increased tRNA loss and exacerbated temperature sensitivity. A similar exacerbated growth defect occurred upon GAAC mutation in *S. cerevisiae trm8Δ* and other single modification mutants that triggered RTD. Thus, these results demonstrate a conserved GAAC activation coincident with RTD in *S. pombe* and *S. cerevisiae*, but an opposite impact of the GAAC response in the two organisms. We speculate that the RTD pathway and its regulation of the GAAC pathway is widely conserved in eukaryotes, extending to other mutants affecting tRNA body modifications.
tRNA modifications are highly conserved and their lack frequently results in growth defects in the yeast *Saccharomyces cerevisiae* and neurologicial disorders in humans. *S. cerevisiae* has two tRNA quality control decay pathways that sense tRNAs lacking modifications in the main tRNA body. One of these, the rapid tRNA decay (RTD) pathway, targets mature tRNAs for 5'-3' exonucleolytic decay by Rat1 and Xrn1. It is unknown if RTD is conserved in eukaryotes, and if it might explain phenotypes associated with body modification defects. Here we focus on *trm8Δ* mutants, lacking m^7^G~46~, in the evolutionarily distant yeast *Schizosaccharomyces pombe*. Loss of m^7^G causes temperature sensitivity and RTD in *S. cerevisiae*, microcephalic primordial dwarfism in humans, and defective stem cell renewal in mice. We show that *S. pombe* *trm8Δ* mutants are temperature sensitive due to tY(GUA) decay by Rat1/Dhp1, implying conservation of RTD among divergent eukaryotes. We also show that the onset of RTD triggers activation of the general amino acid control (GAAC) pathway in both *S. pombe* and *S. cerevisiae*, resulting in further tRNA loss in *S. pombe* and reduced tRNA loss in *S. cerevisiae*. We speculate that RTD and its GAAC regulation will be widely conserved in eukaryotes including humans.

### Introduction

tRNAs are subject to extensive post-transcriptional modifications that often profoundly affect tRNA function, as lack of modifications often leads to growth defects in the budding yeast *Saccharomyces cerevisiae* and to neurological or mitochondrial disorders in humans [1–5]. Many tRNA modifications in the anticodon loop are important for decoding fidelity, reading frame maintenance, and sometimes charging efficiency [6–15]. By contrast, modifications in the tRNA body, the region outside the anticodon loop, are often important for folding and stability [16–18], resulting in substantial growth defects. In *S. cerevisiae*, deletion of *TRM6* or *TRM61* is lethal, associated with lack of 1-methyladenosine at A~58~ (m^1^A~58~) [19], whereas deletion of *TAN1, TRM1, or TRM8* (or *TRM82*) results in temperature sensitivity associated with lack of 4-acetylcytidine at C~12~ (ac^4^C~12~), N_2^-N_2^-dimethylguanosine at G~26~ (m^2,2^G~26~), or 7-methylguanosine at G~46~ (m^7^G~46~) respectively [20–22]. Similarly, human neurological disorders are linked to mutations in *TRMT10A*, associated with reduced 1-methylguanosine at G~9~ (m^1^G~9~) [23,24], *TRMT1* (m^2,2^G~26~) [25–28], *WDR4* (m^7^G~46~) [29–31] and *NSUN2*, associated with reduced 5-methylcytidine (m^5^C) at C~48-50~, as well as at C~34~ and C~46~ [32–34].

In *S. cerevisiae*, lack of any of several tRNA body modifications leads to decay of a subset of the corresponding hypomodified tRNAs, mediated by either of two tRNA quality control pathways, each acting on different hypomodified tRNAs and at different stages of tRNA biogenesis. First, the nuclear surveillance pathway targets pre-tRNA^Met^ lacking m^1^A, acting through the TRAMP complex and the nuclear exosome to degrade the pre-tRNA from the 3’ end [17,35–37]. The nuclear surveillance pathway also targets a large portion of wild type (WT) pre-tRNAs shortly after transcription, ascribed to errors in folding of the nascent tRNA or to mutations arising during transcription [38]. Second, the rapid tRNA decay (RTD) pathway targets a subset of the mature tRNAs lacking m^7^G~46~, m^2,2^G~26~, or ac^4^C~12~, using the 5’-3’ exonucleases Rat1 in the nucleus and Xrn1 in the cytoplasm [18,21,22,39,40]. RTD is inhibited by a *met22Δ* mutation [22,39,41,42] due to accumulation of the Met22 substrate adenosine 3’, 5’ bisphosphate (pAp) [43,44], which binds the active site of Xrn1 and presumably Rat1 [45]. The RTD pathway also targets fully modified tRNAs with destabilizing mutations in the stems, particularly the acceptor and T-stem, which expose the 5’ end [40–42]. The hypomodified tRNAs

### Author summary

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targeted by the RTD pathway also expose the 5’ end, ascribed to destabilization of the tertiary fold [40].

There is limited evidence documenting tRNA quality control decay pathways that act on hypomodified tRNAs in other eukaryotes. A mouse embryonic stem cell line with a knockout of METTL1 (ortholog of S. cerevisiae TRM8) had undetectable m^5G in its tRNA substrates and reduced levels of several METTL1 substrate tRNAs [46]. Similarly, knockdown of METTL1 and NSUN2 (homolog of S. cerevisiae TRM4) in HeLa cells led to reduced levels of tRNA^{Val(AAC)} (abbreviated tV(AAC), as in the Saccharomyces genome database) at 43°C in the presence of 5-fluorouracil (5-FU) [47], a known inhibitor of pseudouridine synthases and 5-methyluridine methyltransferase [48–50]. However, in both of these cases, the underlying mechanism is not known. It was also shown that WT mature tRNAi^{Met} was subject to decay by Xrn1 and Rat1 after 43°C heat shock in HeLa cells, although there was no change in the modification pattern in vivo or in the stability of the tRNA in vitro caused by this temperature shift [51].

The goal of the work described here is to determine if and to what extent tRNA quality control decay pathways are linked to hypomodified tRNAs in eukaryotes other than S. cerevisiae. To address this issue, we have studied the biology of the tRNA m^7G_46 methyltransferase Trm8 in the fission yeast Schizosaccharomyces pombe, which diverged from S. cerevisiae ~ 600 million years ago [52].

We chose to study S. pombe Trm8 because S. cerevisiae trm8Δ mutants were known to trigger decay by the RTD pathway. S. cerevisiae Trm8 forms a complex with Trm82 that is required for formation of m^7G_46 in eukaryotic tRNAs [53,54]. S. cerevisiae trm8Δ and trm82Δ mutants are each modestly temperature sensitive [20], and trm8Δ or trm82Δ mutants also lacking any of several other body modifications had enhanced temperature sensitivity [18]. Moreover, the temperature sensitivity of trm8Δ mutants was suppressed by a met22Δ mutation and was associated with decay of tV(AAC) [22], and the more severe temperature sensitivity of trm8Δ trm4Δ mutants (lacking both m^7G and m^2C) was shown explicitly to be due to RTD of tV(AAC) [18,39]. In addition, in mammalian cells, Trm8 biology has other dimensions of complexity. The human TRM82 ortholog WDR4 was associated with reduced tRNA m^7G modification and a distinct form of microcephalic primordial dwarfism [29]; METTL1 or WDR4 knock out mouse embryonic stem cells showed defects in self renewal and differentiation [46]; and METTL1 was also responsible for m^7G modification of mammalian miRNAs and mRNAs [55,56]. This evidence emphasizes that Trm8/Trm82 (METTL1/WDR4) and/or its m^7G modification product is important in S. cerevisiae and mammals, although the reasons are not yet known beyond S. cerevisiae.

We find here that S. pombe trm8Δ mutants have a temperature sensitive growth defect due primarily to decay of tRNA^{Tyr(GUA)} (tY(GUA)) and to some extent tRNA^{Pro(AGG)} (tP(AGG)) by the Rat1 ortholog Dhp1, demonstrating that a major component of the RTD pathway is conserved between S. pombe and S. cerevisiae. We also find an unexpected connection between the RTD pathway and the general amino acid control (GAAC) pathway in both S. pombe and S. cerevisiae. In both S. pombe trm8Δ mutants and S. cerevisiae trm8Δ trm4Δ mutants, the temperature sensitivity coincides with the onset of tRNA decay, which in turn triggers the GAAC activation, presumably due to the increased stress from the tRNA decay. However, in S. pombe trm8Δ mutants, GAAC activation is deleterious to growth, as mutations in the GAAC pathway restore growth and tRNA levels, whereas in S. cerevisiae trm8Δ trm4Δ mutants, GAAC pathway activation is beneficial, as GAAC mutations exacerbate the growth defect and accelerate tRNA loss. Thus, our results demonstrate a conserved GAAC response associated with tRNA decay by the RTD pathway, but opposite effects on cell physiology in the two organisms. These findings suggest the widespread conservation of the RTD pathway in eukaryotes, and its linkage to the GAAC pathway.
Results

The *S. pombe trm8Δ* mutants lack m^7^G in tRNAs and are temperature sensitive

As Trm8 is the catalytic subunit of the Trm8-Trm82 complex [20], we anticipated that tRNAs from *S. pombe trm8Δ* mutants would lack m^7^G. We purified tY(GUA) and tF(GAA), which had each been previously shown to have m^7^G [57,58], and then analyzed their nucleosides by HPLC analysis. Purified tY(GUA) from *S. pombe trm8Δ* mutants had no detectable m^7^G levels (less than 0.03 moles/mole), compared to near stoichiometric levels in tY(GUA) from WT cells (0.93 +/- 0.22 moles/mole), whereas levels of each of three other analyzed modifications (pseudouridine (Ψ), m^5^C, and m^1^A) were very similar in *trm8Δ* and WT cells (Fig 1A). Similarly, purified tF(GAA) from *trm8Δ* mutants had no detectable levels of m^7^G compared to near stoichiometric levels in WT cells, but otherwise WT levels of Ψ, 2′-O-methylcytidine (Cm) and m^2,2^G (Fig 1A). These results suggest strongly that *S. pombe trm8Δ* is the methyltransferase responsible for m^7^G formation in cytoplasmic tRNAs.

To understand the biology of *S. pombe trm8Δ* mutants, we examined the growth phenotypes of two genetically independent *trm8Δ* mutants. *trm8Δ* mutants were temperature sensitive starting at 37˚C on rich (YES) and minimal (EMM) media, and expression of *P_trm8 trm8Δ* on a plasmid restored WT growth in both media (Fig 1B). Thus, the temperature sensitivity of *trm8Δ* mutants was due to lack of *trm8Δ*.

*S. pombe trm8Δ* mutants have reduced levels of tP(AGG) and tY(GUA) at high temperatures

To determine if the temperature sensitivity of *S. pombe trm8Δ* mutants was associated with tRNA decay, we analyzed tRNA levels of *trm8Δ* mutants after an 8 hour temperature shift in YES media from 30˚C to 36.5˚C, 37.5˚C, and 38.5˚C, which progressively inhibited growth (S1 Fig). We measured tRNA levels of all 21 tRNAs in the Genomic tRNA Database [59] that had a 5-nt variable loop with a central guanosine residue (S1 Table), which is the signature for m^7^G modification [60]. We quantified levels of each tRNA at each temperature relative to the levels of that tRNA in WT cells at 30˚C, after normalization of each to the levels of the non-Trm8 substrate tG(GCC) at the corresponding temperature. We used tG(GCC) as the standard because, for unknown reasons, the usual standards 5S and 5.8S RNA each had temperature-dependent reduction in their levels in *trm8Δ* mutants (S2 Fig), as determined relative to input RNA levels. Note that with tG(GCC) as the standard, the levels of another non-Trm8 substrate, tL(UAA), were also unaffected.

Northern analysis showed that *S. pombe trm8Δ* mutants had significantly reduced levels of two of the 21 potential Trm8 substrate tRNAs as the temperature was increased. The levels of tP(AGG) were substantially reduced in *trm8Δ* mutants, from 70% of the levels in WT cells at 30˚C, to 50%, 31%, and 18% after temperature shift to 36.5˚C, 37.5˚C, and 38.5˚C respectively, whereas levels of tP(AGG) in WT cells remained constant as temperature increased (Fig 2A and 2B). As expected, tP(AGG) is indeed a substrate of Trm8, since purified tP(AGG) from *trm8Δ* mutants had undetectable levels of m^7^G, but WT levels of each of three other modifications (S3 Fig). The levels of tY(GUA) were also reduced in *trm8Δ* mutants as temperature increased, albeit to a lesser extent than tP(AGG) levels. Levels of tY(GUA) in *trm8Δ* mutants were about the same as those in WT cells at 30˚C (119%), remained essentially unchanged at 36.5˚C and 37.5˚C (124%, and 98%), but were reduced to 67% at 38.5˚C, whereas tY(GUA) levels in WT cells were relatively constant at all temperatures. In contrast, none of the 19 other predicted Trm8 substrate tRNAs showed a temperature-dependent reduction in levels in *trm8Δ* mutants (Figs 2A and 2B and S4 and S5). Levels of 15 tRNAs were approximately
constant in trm8Δ mutants as temperature increased, although the initial levels varied somewhat, and levels of the other four tRNAs (tR(CCU), tM(CAU), tV(CAC), and tK(UUU)) were modestly increased at 38.5˚C. Thus, if the temperature sensitivity of trm8Δ mutants was due to loss of tRNAs, the likely candidates were tP(AGG) and tY(GUA).

The growth defect of *S. pombe* trm8Δ mutants is primarily due to loss of tY (GUA)

To evaluate the cause of the temperature sensitivity of *S. pombe* trm8Δ mutants, we analyzed growth after overexpression of tP(AGG) and/or tY(GUA) on leu2+ plasmids (Fig 2C).
Surprisingly, on both YES media and EMM complete (EMMC) media lacking leucine, trm8Δ mutants expressing tY(GUA) grew almost as well as the trm8Δ [P<sub>trm8</sub> trm8<sup>+</sup>] strain or the WT strain at elevated temperatures, whereas trm8Δ mutants expressing two tP(AGG) genes on a plasmid had little effect on the temperature sensitivity (Fig 2D). As expected, northern analysis showed that trm8Δ [leu2<sup>+</sup> tY(GUA)] strains had substantially more tY(GUA) than the trm8Δ [leu2<sup>-</sup>] vector control strain at 30˚C and 38.5˚C (3.2-fold and 6.8-fold more respectively) (S6 Fig). Similarly, trm8Δ mutants expressing two copies of tP(AGG) had more tP(AGG) at 30˚C and 38.5˚C than the vector control, and the levels of the control tT(AGU) were unchanged in all strains at both temperatures. We conclude that although levels of both tY(GUA) and tP(AGG) were reduced in trm8Δ mutants at elevated temperatures in both YES and EMMC media, tY(GUA) is the major physiologically important tRNA for these phenotypes.

Although tY(GUA) overexpression almost completely restored growth of S. pombe trm8Δ mutants in YES and EMMC media at 38˚C and 39˚C, expression of both tY(GUA) and tP(AGG) was required to completely suppress the growth defects in YES + glycerol media (S7 Fig). By contrast, overexpression of tY(GUA) and tP(AGG) had no effect on the known temperature sensitivity of trm8Δ mutants in YES media containing 5-FU [61,62] (S7 Fig), perhaps due to reduced levels of Ψ and 5-methyluridine modifications, which could trigger decay of other hypomodified tRNA species in trm8Δ mutants.

dhp1 mutations suppress the S. pombe trm8Δ growth defect and restore tY(GUA) and tP(AGG) levels

To identify the mechanisms that restore growth to S. pombe trm8Δ mutants at elevated temperatures, we isolated and analyzed spontaneous suppressors of the temperature sensitivity. One major class of four trm8Δ suppressors were as temperature resistant as WT on YES and EMMC media, and nearly as resistant as WT on YES + 5-FU media (Figs 3A and S8). Genome sequencing revealed that these mutants each had distinct missense mutations in the RAT1 ortholog dhp1<sup>+</sup>. The dhp1 mutations each occurred in highly conserved residues, based on an alignment of 18 RAT1/dhp1<sup>+</sup> eukaryotic homologs from multiple phyla (Fig 3B), and are presumably partial loss of function mutations as S. cerevisiae RAT1, is an essential gene [63,64].

Because we obtained four genetically independent S. pombe trm8Δ dhp1Δ mutants and very few other mutations in the whole genome sequencing, it was highly likely that the dhp1 mutations were responsible for the restoration of growth in trm8Δ dhp1Δ mutants. Consistent with this, a plasmid expressing dhp1<sup>+</sup> complemented the S. pombe trm8Δ dhp1-1 suppressor, resulting in temperature sensitivity, but had no effect on WT or trm8Δ mutants (S9 Fig). Thus, we conclude that the dhp1 mutations were responsible for the rescue of growth at high temperature.
As Dhp1 encodes a 5’-3’ exonuclease [65], it seemed highly likely that the *S. pombe trm8Δ dhp1* mutants prevented decay of tY(GUA) and tP(AGG) at non-permissive temperature. Indeed, we found that for each of two dhp1 suppressors, tY(GUA) levels were almost completely restored at 38.5°C, from 29% in the *trm8Δ* mutant to 85% and 82% in the *trm8Δ dhp1-1* and *trm8Δ dhp1-2* mutants respectively (Fig 3C and 3D). Similarly, tP(AGG) levels were virtually completely restored at 38.5°C, from 16% in the *trm8Δ* mutant to 74% and 66% in the *trm8Δ dhp1* suppressors, and the levels of the control tRNA (tP(AGU)) was unaffected. Similar restoration of tY(GUA) and tP(AGG) levels was also observed in the two other *trm8Δ dhp1* suppressors at 38.5°C (S10 Fig). We conclude that, as for RTD in *S. cerevisiae* modification mutants [22,39,40], tRNA decay in *S. pombe trm8Δ* mutants occurs by 5’-3’ exonucleolytic degradation of tRNA, providing strong evidence for conservation of the RTD pathway in *S. pombe*.

**Mutations in the GAAC pathway suppress the *S. pombe trm8Δ* growth defect and restore tRNA levels**

A second major group of six *S. pombe trm8Δ* suppressors was temperature resistant on YES and EMMC media, but sensitive on YES + 5-FU media, and genome sequencing showed that these suppressors each had distinct mutations in elements of the GAAC pathway (Figs 4A and S11). Among these, we found three *trm8Δ* suppressors with *gcn2* mutations, one with a *gcn1* mutation, and two with *tif221* mutations, encoding the translation initiation factor elf2βα (S2 Table). Each of these genes in *S. cerevisiae* is known to be critical for the GAAC pathway [66–68], which is widely conserved in eukaryotes, including *S. pombe* and mammals [69–74]. In this pathway, amino acid starvation leads to uncharged tRNAs that bind Gcn2 to activate its kinase domain, phosphorylation of elf2α by Gcn2, global repression of translation, and derepression of translation of the transcription factor Gcn4, resulting in increased transcription of nearly one tenth of the *S. cerevisiae* genes [66,75–78]. A similar massive transcription program change occurs in *S. pombe* after amino acid starvation [79].

As expected, all *S. pombe trm8Δ* mutants with suppressing mutations in *gcn2*, *gcn1*, or *tif221*, grew poorly on media containing 3-Amino-1,2,4-triazole (3-AT) (S11 Fig), the classical inducer of the GAAC pathway [79–81]. Furthermore, each of two *S. pombe trm8Δ* GAAC suppressors tested (with *gcn2-1* and *tif221-2* mutations) was complemented by re-introduction of the WT gene (Fig 4B), and a re-constructed *trm8Δ gcn2Δ* strain was temperature resistant and sensitive to 3-AT (S12 Fig).

Consistent with their role as *S. pombe trm8Δ* suppressors, all six of the *trm8Δ* GAAC mutants had increased levels of tY(GUA) and tP(AGG) at high temperature. After growth in YES media at 38.5°C, all *trm8Δ* GAAC mutants showed a 1.7-fold to 2.1-fold increase in tY (GUA) levels compared to the parent *trm8Δ* mutant, and tP(AGG) levels were increased ~ 3 fold, whereas the controls tT(AGU) and tV(AAC) did not have increased levels (Figs 4C and 4D and S13). These results provided strong evidence that the effect of the GAAC mutants was to increase tRNA levels to restore growth.
To test if the temperature resistance of *S. pombe* trm8Δ GAAC mutants extended further downstream within the GAAC pathway, we deleted the GAAC transcription factor *fil1+*, the functional equivalent of *S. cerevisiae* Gcn4 [82]. We found that trm8Δ *fil1* mutants were
distinctly more temperature resistant than \textit{trm8Δ} mutants in EMMC-His media, but not as temperature resistant as a \textit{trm8Δ gcn2Δ} mutant (S14 Fig). We thus infer that suppression of the \textit{trm8Δ} temperature sensitivity observed in \textit{S. pombe trm8Δ} GAAC mutants is due in part to transcription activation by Fil1. We note that the \textit{fil1Δ} mutation did not rescue the temperature sensitivity of \textit{trm8Δ} mutants in YES media; this could be due in part to the temperature sensitivity of \textit{fil1Δ} mutants in YES media.

### The temperature sensitivity of \textit{S. pombe trm8Δ} mutants coincides precisely with the onset of GAAC activation and tY(GUA) decay

Since \textit{S. pombe trm8Δ} suppressors in different components of the GAAC pathway all restored tY(GUA) and tP(AAG) levels, we inferred that \textit{trm8Δ} mutants activated the GAAC pathway at non-permissive temperatures, and that this activation somehow promoted further loss of the tRNA. To establish the precise connection between growth, tRNA levels, and GAAC activation at non-permissive temperatures, and that this activation somehow promoted further loss of the \textit{trm8} mutant (S14 Fig). We thus infer that suppression of the \textit{trm8Δ} growth defect. Alternatively, GAAC activation could be a consequence of both tY(GUA) and tP(AAG) decay, reduced tRNA charging associated with \textit{trm8Δ} mutants at high temperatures.

As tY(GUA) was the major physiologically relevant substrate in YES media (Fig 2D), we speculated that at 38.5˚C, tY(GUA) decay might be driving the GAAC activation associated with the \textit{trm8Δ} growth defect. Alternatively, GAAC activation could be a consequence of both tY(GUA) and tP(AAG) decay, reduced tRNA charging associated with \textit{trm8Δ} mutants at high
temperature, or partly as a consequence of temperature stress itself, as a number of different stress conditions are known to activate the GAAC pathway [72,74,84,85].

To determine the extent to which reduced tRNA levels activated the GAAC pathway, we examined GAAC induction of *S. pombe* *trm8Δ* strains after overproduction of tY(GUA) and tP(AGG), using the same samples we used to show that overproduction of tY(GUA) suppressed the *trm8Δ* temperature sensitivity (Figs 2D and S6). As expected, relative lys4* mRNA levels were increased in *trm8Δ* [vector] strains grown at 38.5°C, compared to this strain at 30°C (4.2 vs 0.57, 7.4-fold) or to WT at 38.5°C (7.0 fold), indicating GAAC activation (Fig 5D). Notably, relative lys4* mRNA levels were reduced 4.0-fold in the *trm8Δ* [tY(GUA)] strain compared to the corresponding *trm8Δ* [vector] strain (from 4.2 to 1.04), and were not reduced in the *trm8Δ* [tP(AGG)] strain. Based on these results, we conclude that reduced function of tY(GUA) is the primary cause of GAAC activation and temperature sensitivity of *trm8Δ* mutants at 38.5°C. As charging of tY(GUA) was distinctly but marginally reduced at 38.5°C relative to WT (S18 Fig), we cannot determine if the GAAC activation was due to reduced levels of tY(GUA), or to a combination of reduced levels and charging [69,76,80,83,86].

To determine the effects of *Trm8* and *Gcn2* on processing and transcription, we examined expression of the four pre-tY(GUA) species (1–1, 1–2, 1–3, and 2, as described [59]) in the WT, *trm8Δ*, and *trm8Δ gcn2-1* strains, using appropriate intron-specific probes. With a probe specific for the pre-tY(GUA)-2 species, we found that at all temperatures *trm8Δ* mutants accumulated significantly more of the 3’ end-extended and the end-matured pre-tY(GUA) species than the WT strains (S19 Fig). Similarly, with the pre-tY(GUA)-1-3 probe, we observed accumulation of the end-matured pre-tY(GUA) in *trm8Δ* mutants at all temperatures. These results suggest a processing defect due to lack of m7G for these pre-tY(GUA) species. We also found that levels of the primary transcript, corresponding to the largest pre-tY(GUA) species, were slightly elevated in *trm8Δ gcn2-1* mutants relative to *trm8Δ* mutants, at both 37.5°C and 38.5°C, but not at 36.5°C (S19A and S19B Fig). This result suggests that tRNA transcription might play some role in restoring tY(GUA) levels in a *trm8Δ gcn2-1* mutant at 37.5°C and 38.5°C, although it is not clear yet if this effect accounts for all of the suppression.

In *S. cerevisiae*, mutation of the GAAC pathway exacerbates the effects of the RTD pathway

To investigate the evolutionary implications of the GAAC pathway on RTD, we examined the consequences of deletion of GAAC components in *S. cerevisiae* *trm8Δ trm4Δ* mutants, which are highly temperature sensitive due to substantial decay of tV(AAC) by the RTD pathway, compared to the modest RTD-dependent temperature sensitivity and the limited tV(AAC) decay of *S. cerevisiae* *trm8Δ* mutants [18,39]. In contrast to our results in *S. pombe* *trm8Δ*
GAAC mutants, deletion of GCN1 or GCN2 exacerbated the temperature sensitivity of S. cerevisiae trm8Δ trm4Δ mutants in both rich (YPD) and minimal complete (SCD) media, and this exacerbated temperature sensitivity was also observed upon deletion of the GAAC transcription factor GCN4 (Fig 6A). Moreover, we found that a met22Δ mutation, known to prevent RTD, reversed the enhanced temperature sensitivity of a trm8Δ trm4Δ gcn2Δ strain relative to a trm8Δ trm4Δ mutant (S20 Fig). Furthermore, a similar exacerbated temperature sensitivity due to mutation of the GAAC pathway was also observed in other S. cerevisiae modification mutants known to be subject to RTD [22,39], including trm8Δ, trm1Δ, tan1Δ, and tan1Δ trm4Δ mutants (S21 Fig).

To determine if the exacerbated growth defect of S. cerevisiae trm8Δ trm4Δ GAAC mutants was due to exacerbated loss of tV(AAC), we analyzed tRNA levels after a four-hour temperature shift from permissive to non-permissive temperature (27˚C to 32˚C). Consistent with the exacerbated temperature sensitivity caused by the gcn1Δ and gcn2Δ mutations, tV(AAC) levels were further reduced in both trm8Δ trm4Δ gcn1Δ and trm8Δ trm4Δ gcn2Δ mutants at 32˚C, compared to the trm8Δ trm4Δ mutant (12% and 17% vs 43%, relative to the values at 27˚C) (Fig 6B and 6C). Using a pre-tV(AAC) probe specific for 7 of the 14 tV(AAC) genes in S. cerevisiae, we found that levels of the primary pre-tV(AAC) transcript were modestly reduced at 32˚C in the trm8Δ trm4Δ gcn1Δ mutant, relative to the trm8Δ trm4Δ mutant (S22 Fig). This result suggests that reduced pre-tV(AAC) transcription could be responsible for some of the exacerbated loss of tV(AAC) in the trm8Δ trm4Δ gcn1Δ mutant and for its exacerbated temperature sensitivity, although the magnitude of the change may not account for all of the additional loss of tV(AAC).

The temperature sensitivity and tV(AAC) decay of S. cerevisiae trm8Δ trm4Δ mutants coincides with GAAC activation

Since GAAC mutations exacerbated the RTD growth defect and enhanced the decay of an S. cerevisiae trm8Δ trm4Δ mutant at 32˚C, it seemed likely that the GAAC pathway was activated in the trm8Δ trm4Δ mutant. To evaluate GAAC activation, we measured mRNA levels of the Gcn4 target genes LYS1 and HIS5 after the 4 hour temperature shift of trm8Δ trm4Δ mutants to 32˚C, using the same RNA as in the northern analysis of decay (Figs 6B and 6C and S22).

RT-qPCR analysis showed that trm8Δ trm4Δ mutants had a large increase in relative LYS1 mRNA levels at 32˚C, compared to 27˚C (22.2 vs 0.81, 27.4-fold), or to WT cells at either 27˚C or 32˚C (Fig 6D), showing that the GAAC activation was specific to the trm8Δ trm4Δ mutant at 32˚C. We observed a similar activation of the GCN4 target HIS5 in the trm8Δ trm4Δ mutant at 32˚C. This GAAC activation was correlated with a modest but distinct increase in uncharged tV(AAC) commensurate with the reduced tV(AAC) levels (S23 Fig). Furthermore, we found that overproduction of tV(AAC) in trm8Δ trm4Δ mutants suppressed induction of the GAAC pathway (Fig 6E), showing that GAAC activation in trm8Δ trm4Δ mutants was due to the reduced function of tV(AAC). Thus, in both S. cerevisiae trm8Δ trm4Δ mutants and S. pombe trm8Δ mutants, degradation of a single biologically relevant tRNA is the cause of GAAC induction, which then either promotes further loss of tRNA in S. pombe, or restores tRNA in S. cerevisiae (Fig 7).

Discussion

The results described here provide strong evidence that the RTD pathway is conserved between the distantly related species S. cerevisiae and S. pombe. We have shown that the temperature sensitivity of S. pombe trm8Δ mutants is due to reduced levels of tY(GUA) and to some extent tP (AGG), and is efficiently suppressed by mutations in the 5’-3’ exonuclease Dhp1 that
Fig 6. Mutations in the GAAC pathway exacerbate the temperature sensitivity of *S. cerevisiae trm8Δ trm4Δ* mutants as well as tV(ACC) decay. (A) Deletion of *GCN1*, *GCN2*, or *GCN4* exacerbated the temperature sensitivity of *S. cerevisiae trm8Δ trm4Δ* mutants in YPD and SDC media. Strains were
grown overnight in YPD media at 27°C and analyzed for growth on YPD and SDC plates at different temperatures. (B) Deletion of GCN1 or GCN2 exacerbated tV(AAC) decay of trm8Δ trm4Δ mutants at 32°C. Strains were grown in YPD media 27°C, shifted to 32°C and harvested after 4 hours, and then bulk RNA was isolated and analyzed by northern blotting. (C) Quantification of tRNA tV(AAC), tr(UCU), and tW(CCA) levels from the northern in Fig 6B. The bar chart depicts levels of tRNA species at 27°C or 32°C, relative to levels of that tRNA in the WT strain at 27°C (each value itself first normalized to levels of the control 5S rRNA). tRNA levels are indicated by diagonal hatching for WT (green);  exacerbat ed tV(AAC) decay of grown overnight in YPD media at 27°C and analyzed for growth on YPD and SDC plates at different temperatures. (C) Quantification of tRNA tV(AAC), tr(UCU), and tW(CCA) levels from the northern in Fig 6B. The bar chart depicts levels of tRNA species at 27°C or 32°C, relative to levels of that tRNA in the WT strain at 27°C (each value itself first normalized to levels of the control 5S rRNA). tRNA levels are indicated by diagonal hatching for WT (green);  exacerbat ed tV(AAC) decay of grown overnight in YPD media at 27°C and analyzed for growth on YPD and SDC plates at different temperatures. (C) Quantification of tRNA tV(AAC), tr(UCU), and tW(CCA) levels from the northern in Fig 6B. The bar chart depicts levels of tRNA species at 27°C or 32°C, relative to levels of that tRNA in the WT strain at 27°C (each value itself first normalized to levels of the control 5S rRNA). tRNA levels are indicated by diagonal hatching for WT (green); 

6B was used for RT-qPCR analysis of levels of LYS1 mRNA, normalized to ACT1. mRNA levels are indicated by horizontal lines for LYS1 and 

hatching for WT (green); Sc trm8Δ (brown); Sc trm8Δ gcn1Δ (light blue); and Sc trm8Δ trm4Δ gcn1Δ (dark blue). (D) trm8Δ trm4Δ mutants induced the GAAC pathway at 32°C. Bulk RNA from the growth done for Fig 6B was used for RT-qPCR analysis of levels of LYS1 and HIS5 mRNA, normalized to ACT1. mRNA levels are indicated by horizontal lines for LYS1 and 

hatching for HIS5, WT (green); Sc trm8Δ (brown). Right side: Relative levels of LYS1 mRNA of WT cells grown at 30°C in SD-His media, and then treated with 10 mM 3-AT for 1 hour, evaluated in parallel to other samples. (E) tV(AAC) overproduction repressed the GAAC induction of trm8Δ trm4Δ mutants at 36°C. Strains with plasmids as indicated were grown in SD-Ura media 27°C and shifted to 36°C for 1 hour, and then RNA was isolated and relative 

LYS1 mRNA levels were analyzed by RT-qPCR as described in Fig 6D. Right side: GAAC induction of WT cells grown and induced with 3-AT as described in 

Fig 6D. 

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concomitantly restore the levels of these tRNAs, strongly suggesting decay of tY(GUA) and tP(AGG) by the RTD pathway. As RTD is triggered in S. cerevisiae strains lacking m3A2G26 or ac4C12, as well as in strains lacking m7G46s, [22,39], we speculate that the RTD pathway will also act in S. pombe strains lacking other body modifications. Furthermore, given the large evolutionary distance between S. cerevisiae and S. pombe, we speculate that the RTD pathway is conserved throughout eukaryotes. The existence of a mammalian RTD pathway could explain the reduced levels of specific tRNA species in mouse strains lacking m7C in their tRNAs [33,87] and in mouse embryonic stem cells lacking m7G in their tRNAs [46], and might explain other phenotypes associated with mutations in METTL1 or WDR4 [29,30,46,88].

It is puzzling that although tP(AGG) is substantially more degraded than tY(GUA) in S. pombe trm8Δ mutants at elevated temperatures, the temperature sensitivity of the mutants in both rich and minimal media is primarily due to decay of tY(GUA). One possible explanation of this result is that tY(GUA) levels might be more limiting in the cell than tP(AGG) levels at elevated temperature, relative to the number of their respective cognate codons requiring decoding. This type of argument was advanced as a possible explanation for why the growth defects of tA-Lacking S. pombe tit1Δ mutants were rescued by increased expression of tY(GUA), but not by any of the other four Tit1 tRNA substrates [89]. A second, and less likely, interpretation is that tP(AGG) decoding might be compensated by other tRNA Pro isodecoders specific for the CCN codon box. This explanation is based on the finding that in S. cerevisiae, deletion of the two tP(AGG) genes is viable, implying that the 10 tP(UGG) isodecoders can decode all four proline CCN codons [90]. However, as S. pombe has six tP(AGG) genes and only two tP(UGG) genes (as well as one tP(CGG)), it seems unlikely that the loss of almost all of the tP(AGG) in trm8Δ mutants can be efficiently compensated by the small number of tP(UGG) species (assuming that tRNA expression from each gene is comparable).

It is not immediately clear why tP(AGG) and tY(GUA) are the specific tRNAs subject to RTD in S. pombe trm8Δ mutants. Based on current understanding of RTD determinants in S. cerevisiae, RTD substrate specificity is determined by stability of the stacked acceptor and T-stem, with contributions to stability from the tertiary fold that are reduced in modification mutants, and some contributions from the other two stems, but not the anticodon loop [18,22,39–42]. tP(AGG) may be an RTD substrate because it is predicted to have a less stable acceptor and T-stem than most Trm8 substrates (S1 Table) [91]. Furthermore, the destabilizing C4-A68 mismatch in the middle of the tP(AGG) acceptor stem might be expected to lead to increased local breathing at the 5’ end, which is likely important for recognition by the 5’-3’ exonucleases Xrn1 and Rat1, as the Xrn1 active site binds the three most 5’ nucleotides [92]. However, it is more difficult to rationalize why tY(GUA) is a substrate for RTD in trm8Δ mutants, as its acceptor and T-stem are predicted to be moderately stable among Trm8 sub-

strates. However, tY(GUA) does have a destabilizing N27-N43 pair (C27-U43 for 3 isodecoders,
and U_{27}–U_{43} for one isodecoder), which might reduce the stability of the tertiary fold by affecting stability of the adjacent tertiary 26–44 interaction [93–95].

It is not yet clear how Dhp1 degrades tY(GUA) in S. pombe trm8Δ mutants. In S. cerevisiae, Rat1 is nuclear [96] and catalyzes a substantial amount of the decay of mature tV(AAC) in trm8Δ trm4Δ mutants [39], suggesting that the retrograde transport pathway is required to
deliver the tV(AAC) substrate to the nucleus [5,97–100]. However, we do not know the exact species of tY(GUA) that is degraded by Dhp1 in S. pombe trm8Δ mutants. If mature tY(GUA) is the actual Dhp1 substrate, it is almost certainly subject to retrograde transport back to the nucleus for the subsequent decay, because Dhp1 is known to be nuclear [64,101] and S. pombe pre-tRNA splicing initiates in the cytoplasm on the mitochondrial surface [102]. Such a retrograde transport mechanism for hypomodified tY(GUA) lacking m^5G_26 in S. pombe trm8Δ mutants would be similar to that shown for hypomodified tRNAs lacking m^7A_26 in S. cerevisiae trm1Δ mutants [100]. However, it is also possible that Dhp1 acts to degrade unspliced pre-tY(GUA) that accumulates in trm8Δ mutants, analogous to the recently described Met22-dependent pre-tRNA decay pathway [103].

It seems likely that the 5-FU sensitivity of S. pombe trm8Δ mutants is due to decay of multiple tRNA species in the presence of the drug, caused by the reduced levels of Ψ and m^5U [48–50], in addition to the lack of m^3G. This interpretation is consistent with the lack of suppression of the 5-FU sensitivity of trm8Δ mutants by tY(GUA) and tP(AGG), and its almost complete suppression by dhp1 mutations, and is consistent with the enhanced 5-FU sensitivity of a number of tRNA body modification mutants in S. cerevisiae [61].

Our finding that loss of function of tRNA due to tRNA decay is itself the trigger for induction of the GAAC response in both S. pombe trm8Δ mutants and S. cerevisiae trm8Δ trm4Δ mutants suggests an intimate relationship between reduced tRNA function and GAAC activation. The loss of functional tRNA below some presumed threshold level is the proximal cause of GAAC induction, because in each organism the GAAC pathway is activated at the lowest temperature at which tRNA decay and a growth defect is observed, and in each organism overproduction of the physiologically relevant tRNA represses GAAC induction. The GAAC pathway has previously been implicated in the biology of a number of anticodon loop modifications [83,104,105]. Robust constitutive GAAC induction is observed in S. cerevisiae and S. pombe trm7Δ mutants (lacking Nm_{32} and Nm_{34}) and S. cerevisiae pu3Δ mutants (lacking Ψ_{38} and Ψ_{50}) [83,105], each of which has a constitutive growth defect [106,107], and GAAC induction is known to be Gcn2-dependent in S. cerevisiae trm7Δ mutants [83]. By contrast, S. cerevisiae mutants lacking either the mcm^5U or the s^2U moiety of mcm^5s^2U induce the GAAC pathway independently of Gcn2 at 30˚C [104] and are temperature sensitive at 37˚C [108]. Our finding that S. pombe trm8Δ and S. cerevisiae trm8Δ trm4Δ mutants each trigger Gcn2-dependent GAAC induction only at the temperature that the growth defect is observed is consistent with Gcn2-dependent GAAC induction in S. cerevisiae anticodon loop modification mutants with a constitutive growth defect.

It is striking that the induction of the GAAC response due to tRNA decay in S. pombe trm8Δ mutants and S. cerevisiae trm8Δ trm4Δ mutants has opposite consequences in each organism. In S. pombe trm8Δ mutants, activation of the GAAC response exacerbates the growth defect, as mutation of any of four components (gcn1, gcn2, tf221, or fil1) protects against loss of tRNA. Activation of the GAAC pathway is also part of the reason that S. cerevisiae trm7Δ mutants grow poorly [83], and defects in the integrated stress response pathway (ISR) in humans are implicated in disease phenotypes [109–111]. By contrast, in S. cerevisiae trm8Δ trm4Δ mutants, activation of the GAAC response rescues the growth defect, as deletion of any of three GAAC components (gcn1Δ, gcn2Δ, or gcn4Δ) exacerbates the growth defect. Furthermore, this result extends to multiple S. cerevisiae modification mutants with an RTD phenotype, since a gcn2Δ mutation also exacerbated the growth defects of trm8Δ, trm1Δ, tan1Δ, and tan1Δ trn44Δ mutants. Although the rescue of RTD in S. cerevisiae by GAAC activation is opposite to the exacerbating effect of GAAC activation in S. pombe trm8Δ mutants, it is consistent with a concerted stress response. Moreover, the finding that GAAC effects on RTD extended to fil1Δ/ gcn4Δ mutations in S. pombe and S. cerevisiae has mechanistic implications. Deletion of GCN1 or GCN2 each prevent sensing of tRNA status, and the consequent eIF2α phosphorylation,
reduced translation initiation, reduced global translation, and massive downstream transcription activation. However, as fill+/Gcn4 is downstream of the sensing machinery, but upstream of the transcription activation, we infer that the GAAC effects on RTD in S. pombe and S. cerevisiae are in part due to lack of transcription activation by fill+/Gcn4.

The opposite effects of GAAC activation on growth and RTD in S. pombe tram8Δ mutants and in several S. cerevisiae RTD mutants is likely due to a differential GAAC response. Although in each organism the GAAC pathway is known to regulate the transcription of more than 500 genes [75,77,79,82], there are distinct differences in the GAAC response in the two species. For example, it is known that the GAAC response to amino acid starvation results in repression of methionine synthesis genes in S. pombe but induction of these genes in S. cerevisiae [79]. As a met22Δ mutation is known to inhibit RTD in S. cerevisiae [22,39], this opposite GAAC activation effect on methionine genes in the two organisms is in the wrong direction to explain the opposite RTD effects of the GAAC pathway. Other possible explanations for the differential effects of the GAAC pathway on RTD include differential regulation of the synthesis or biochemical activity of RTD regulators such as EF1A, aminoacyl tRNA synthetases, pol III transcription [22,112], or 5′-3′ exonucleases [39,42], as well as changes in any number of indirect effectors affecting overall levels or availability of tRNA and/or nucleases. In addition, the overall stress response pathways are substantially different between S. cerevisiae and S. pombe. In S. cerevisiae, Gcn2 is the sole eIF2α kinase regulating stress responses [78,113,114], whereas in S. pombe three different eIF2α kinases (Gcn2, Hri1 and Hri2) [115] each respond to a diverse set of stress treatments [72,74,84,85,109]. The differences in kinases affecting eIF2α phosphorylation implies substantial differences between the two species in regulation of all sorts of combinations of stress response, which might be occurring at elevated temperature when tRNA decay is occurring [116].

The results outlined here underscore that GAAC activation occurs in S. cerevisiae and S. pombe tram8Δ modification mutants precisely at the point of observed growth stress due to tRNA decay, albeit with different effects in S. pombe tram8Δ mutants and S. cerevisiae tram8Δ tram4Δ mutants. These results, coupled with the constitutive GAAC activation in S. pombe and S. cerevisiae tram7Δ mutants and S. cerevisiae pus3Δ mutants [83,105], fuel speculation that the GAAC response will also be activated in mammals and other eukaryotes with tRNA modification mutations or other mutations that result in reduced tRNA function. Given that GAAC activation at the onset of reduced tRNA function regulates RTD in opposite ways in S. pombe and S. cerevisiae, it would be interesting to determine the GAAC effect on tRNA decay and tRNA levels in mammalian systems. Based on the observation that GAAC activation in mice attenuates the growth defects caused by the combination of reduced tRNA<sub>Arg(UCU)</sub> levels and a defect in the ribosome recycling component GTPBP2 [86], we speculate that GAAC activation by reduced tRNA function in mammals will likewise attenuate RTD and promote survival.

As Trm8 is phosphorylated and likely inactivated by treatment of HEK293 cells with insulin-like growth factor-1 [117], it seems plausible that if RTD is conserved in mammals, m<sup>G</sup> modification dynamics could be used to regulate tRNA levels physiologically. There is growing evidence that levels of a number of modifications are under dynamic control in different conditions [118,119]. There is also evidence that regulation of tRNA expression plays an important role in differentiation and proliferation, and is also a characteristic of breast cancer [120–124]. For example, tRNA<sup>Arg</sup> iso-acceptors have different expression in differentiated vs proliferating cells [123] and tR(UCU) iso-decoders show tissue specific regulation of expression [125]. It remains to be determined if regulated changes in expression, phosphorylation, or biochemical activity of METTL1 or WDR4 result in altered m<sup>G</sup> levels and consequent changes in tRNA levels that physiologically regulate expression.
Materials and methods

Yeast strains

*S. pombe* haploid WT and two independent *S. pombe* trmΔ::kanMX strains were derived from SP286 (ade6-M210/ade6-M216, leu1-32/leu1-32, ura4-D18/ura4-D18 h+/h+) [126], and were obtained from Dr. Jeffrey Pleiss. *S. pombe* trmΔ::kanMX strains were also generated from haploid WT strains by PCR amplification of trmΔ::kanMX DNA, followed by linear transformation using lithium acetate [127]. *S. cerevisiae* deletion strains are shown in S3 Table, and were constructed by linear transformation with PCR amplified DNA from the appropriate knockout strain [128]. All strains were confirmed before use by PCR amplification.

Plasmids

Plasmids used in this study are listed in S4 Table. AB553-1 was constructed by insertion of a NotI restriction site between the Pnmt1 promoter and the PstI site of the pREP3X plasmid. The *S. pombe* plasmid expressing *S. pombe* Ptrm8+ trm8+ (ETD 67–1) was constructed by inserting PCR amplified DNA genomic DNA (including 1000 bp upstream and 1000 bp downstream) into the NotI and XhoI sites of AB 553–1, removing the Pnmt1 promoter. Plasmids expressing *S. pombe* Pdhp1 dhp1+ or *S. pombe* tRNA genes were constructed using the same approach, including ~ 300 bp upstream and 300 bp downstream for the tRNA genes. *S. pombe* plasmids expressing Pnmt1: gcn2+ (low strength, no message in thiamine) or Pnmt1: tif221+ (medium strength, no message in thiamine) were constructed by PCR amplification of the respective coding sequence from *S. pombe* WT genomic DNA (including introns), and insertion into the XhoI and BamHI sites of the pREP81X or pREP41X vectors respectively.

Yeast media and growth conditions

*S. pombe* strains were grown at desired temperatures in rich (YES) media (containing 0.5% yeast extract, 3% glucose, and supplements of 225 mg/l of adenine, uracil, leucine, histidine and lysine), or Edinburgh minimal media (EMM) containing glucose and the same supplements, as well as similar amounts of relevant auxotrophic requirements. Minimal complete (EMM-C) media was supplemented with 225 mg/l of all amino acids, adenine, and uracil, as well as 100 mg/l of para-amino benzoic acid and inositol, and 1125 mg/l of leucine for Leu- auxotrophs [79]. For temperature shift experiments, cells were grown in YES or EMMC media at 30°C to OD600 ~ 0.5, diluted to ~ 0.1 OD in pre-warmed media at the desired temperature, grown to OD ~ 0.5, harvested at 4°C, washed with ice cold water, frozen on dry ice, and stored at -80°C. To select spontaneous suppressors of *S. pombe* trmΔ mutants, cells were grown overnight in YES media at 30°C and ~107 cells were plated on YES media plates at 38°C and 39°C. *S. cerevisiae* strains were grown in rich (YPD) media (containing 1% yeast extract, 2% peptone, 2% dextrose, and 80 mg/l adenine hemisulfate), or minimal complete (SDC) media [129] as indicated, and temperature shift experiments were performed as described for *S. pombe*. All experiments with measurements were performed in biological triplicate, unless otherwise noted.

Bulk RNA preparation and northern blot analysis

For northern analysis, 2 or 3 biological replicates were grown in parallel, and then bulk RNA was isolated from ~ 3–5 OD pellets using glass beads and phenol [130] (for *S. pombe*) or hot phenol (for *S. cerevisiae*), resolved on a 10% polyacrylamide (19:1), 7M urea, 1X TBE gel, transferred to Amersham Hybond-N+ membrane, and analyzed by hybridization to 5’ 32P-labeled DNA probes (S5 Table) as described [18]. For analyzing tRNA charging levels of both *S.
*Pombe* and *S. cerevisiae*, RNA was prepared under acidic conditions (pH 4.5), resolved on a 6.5% polyacrylamide (19:1), 8 M urea, 0.1 M sodium acetate (pH 5.0) gel at 4˚C, and analyzed as described. [18].

**Quantitative RT-PCR analysis**

Strains were grown in triplicate to log phase and bulk RNA was prepared from 2–5 OD pellets using acid washed glass beads and phenol. Then, RNA was treated with RQ1 RNase-free DNase (Promega), reverse transcribed with Superscript II Reverse Transcriptase, and quantitative PCR was performed on the cDNA as previously described [131].

**Isolation and purification of tRNA**

*S. pombe* WT and trm8Δ mutant strains were grown to ~ 0.5 OD in YES media at 30˚C. Then bulk low molecular weight RNA was extracted from ~ 300 OD of pellets by using hot phenol, and tRNAs were purified using 5’-biotinylated oligonucleotides complementary to the corresponding tRNAs (S6 Table) as previously described [132].

**HPLC analysis of nucleosides of purified tRNA**

Purified tRNAs (~ 1.25 μg) were digested to nucleosides by treatment with P1 nuclease, followed by phosphotase, as previously described [132], and nucleosides were analyzed by HPLC at pH 7.0 as previously described [133].

**Whole genome sequencing**

Whole genome sequencing was performed by the University of Rochester Genomics Center at a read depth of 20–110 per genome nucleotide.

**Crude extracts and western blot analysis**

Crude extracts of *S. pombe* WT and trm8Δ mutants were prepared by lysis with glass beads as described [79]. Then 25 μg of crude extract proteins were resolved on 4–20% SDS-PAGE gels (Criterion TGX, Bio-Rad), transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad), and probed with antibodies as described [134], using anti-phosphorylated eIF2α (Cat. # 44-728G, Thermofisher; diluted 1:6000) and anti-α-tubulin (Cat. # T-5168 Sigma, diluted 1:6000).

**Supporting information**

S1 Fig. *S. pombe* trm8Δ mutants have a temperature sensitive growth defect in liquid YES media. Strains were grown in YES media at 30˚C, shifted to the indicated temperatures, and then growth was monitored for 8 hours before harvest as described in Materials and Methods, and tRNA analysis as done in Fig 2A and 2B. WT, green; Sp trm8Δ, brown. (PDF)

S2 Fig. *S. pombe* trm8Δ mutants had reduced 5S rRNA and 5.8S rRNA levels at higher temperatures. The northern blot shown in Fig 2A was used to analyze the non-Trm8 substrate tL (UAA), 5S rRNA, and 5.8S rRNA. The bar chart depicts levels of RNA species at each temperature, relative to their levels in the WT strain at 30˚C (each value itself first normalized to levels of the control non-Trm8 substrate tG(GCC)). 30˚C, green; 36.5˚C, yellow; 37.5˚C, orange; 38.5˚C, red. (PDF)
S3 Fig. tP(AGG) of *S. pombe* trm8Δ mutants has no detectable m7G. *trm8Δ* mutants and WT cells were grown in YES media at 30˚C and tP(AGG) was purified and analyzed for modifications as in Fig 1A.

(PDF)

S4 Fig. Northern analysis of all Trm8 substrates (except tP(AGG), tY(GUA), and tT(AGU)) in *S. pombe trm8Δ* and WT cells after shift from 30˚C to 36.5˚C, 37.5˚C, and 38.5˚C. The northern blot shown in Fig 2A was continued to analyze levels of all other predicted Trm8 substrate tRNAs, as well as the non-Trm8 substrate tL(UAA), 5S RNA, and 5.8S RNA, in WT and *trm8Δ* mutants at different temperatures.

(PDF)

S5 Fig. A. Among 21 predicted Trm8 substrate tRNAs, only tP(AGG) and tY(GUA) had reduced levels in *S. pombe* trm8Δ mutants at elevated temperatures. tRNA levels were quantified, relative to tG(GCC), as described in Fig 2B. Note that data from Fig 2B is also included here for completeness. B. Analysis of Trm8 substrate tRNAs in WT cells at elevated temperatures.

(PDF)

S6 Fig. A. Overproduction of tY(GUA) and tP(AGG) resulted in increased levels of the corresponding tRNAs in *S. pombe* trm8Δ mutants and WT cells. Strains with plasmids as indicated were grown in EMMC-Leu media at 30˚C and shifted to 38.5˚C for 8 hours, and then RNA was isolated and analyzed by northern blotting as in Fig 2A. B. Quantification of tRNA levels in *S. pombe* trm8Δ mutants and WT cells overproducing tY(GUA) or tP(AGG). Quantification was done as in Fig 2B.

(PDF)

S7 Fig. Overproduction of both tY(GUA) and tP(AGG) fully restored growth of *S. pombe* trm8Δ mutants in YES + glycerol media, but not in YES media containing 5-FU. Strains grown for Fig 2D were analyzed for growth on plates containing YES media with 3% glycerol (instead of 3% glucose) and YES media with 5-FU (30 μg/ml).

(PDF)

S8 Fig. *dhp1* mutations restored growth of *S. pombe* trm8Δ mutants in YES + 5-FU media. Strains grown for Fig 3A were analyzed for growth on YES + 5-FU (30 μg/ml) plates.

(PDF)

S9 Fig. Expression of P^dhp1^ dhp1^+ restored temperature sensitive growth in the *S. pombe* trm8Δ dhp1-1 mutant. WT, trm8Δ, and trm8Δ dhp1-1 cells expressing P^dhp1^ dhp1^+ or a vector were grown overnight in EMMC-Leu media at 30˚C, and analyzed for growth.

(PDF)

S10 Fig. A. *S. pombe* trm8Δ dhp1-3 and trm8Δ dhp1-4 mutants also restored tY(GUA) and tP(AGG) tRNA levels at 38.5˚C. Strains were grown in YES media at 30˚C and shifted to 38.5˚C for 8 hours, and RNA was isolated and analyzed by northern blotting as in Fig 2A. B. Quantification of tRNA levels in different *S. pombe* trm8Δ dhp1 mutants. tRNA levels were quantified as in Fig 2B. tT(AGU), brown; tP(AGG), purple; tY(GUA), gray.

(PDF)

S11 Fig. Mutations in the GAAC pathway did not rescue the 5-FU sensitivity of *S. pombe* trm8Δ mutants, and conferred enhanced 3-AT sensitivity. Strains grown for Fig 4A were analyzed for growth on plates containing YES media + 5-FU (30 μg/ml) and EMMC-His.
media + 10 mM 3-AT.

(S12 Fig) Reconstructed *S. pombe trm8Δ gcn2Δ* mutants had the same growth properties as the original *trm8Δ gcn2-1* strain. Strains were analyzed for growth on YES media, EMM-C-His or EMMC-His media containing 10 mM 3-AT, as described in Fig 1B. Reconstructed *S. pombe trm8Δ* mutant was labeled as V3.

(S13 Fig) A. Northern analysis of WT, *S. pombe trm8Δ, trm8Δ gcn2-2, trm8Δ tif221-1*, and *trm8Δ gcn2-3* cells. Strains were grown in YES media at 30˚C and shifted to 38.5˚C for 8 hours, and RNA was isolated and analyzed by northern blotting as in Fig 2A. B. Quantification of tRNA levels. tRNA levels were quantified as in Fig 2B. n = 2 for all strains.

(S14 Fig) Deletion of *fil1*+ partially restored growth in *S. pombe trm8Δ* mutants in EMM-C-His media. Strains were grown overnight in YES media at 30˚C and analyzed for growth.

(S15 Fig) The temperature sensitivity of *S. pombe trm8Δ* mutants observed at 38.5˚C in YES liquid media was suppressed in a *trm8Δ gcn2-1* mutant. Strains were grown in YES media at 30˚C, shifted to 36.5˚C, 37.5˚C, and 38.5˚C as indicated, and then growth was monitored for 8 hours before harvest as described in Materials and Methods, and analysis of mRNAs and tRNAs in Figs 5A–5C and S16 and S19.

(S16 Fig) *S. pombe trm8Δ* mutants induced *aro8*+ mRNA expression at 38.5˚C, but not at 37.5˚C. Bulk RNA from the growth in S15 Fig was used for the RT-qPCR analysis of *aro8*+ (SPAC56E4.03) mRNA levels, as in Fig 5A.

(S17 Fig) A. *S. pombe trm8Δ* mutants had increased levels of phosphorylated eIF2α at 38.5˚C WT and *S. pombe trm8Δ* strains were grown in YES media at 30˚C, shifted to 38.5˚C for 8 hours and cells were harvested. Then crude extracts were prepared and analyzed by western blotting as described in Materials and Methods, using anti-phosphorylated eIF2α and anti-α-tubulin. Controls: WT and gcn2Δ mutants were grown at 30˚C in EMMC-His media and, where indicated, treated with 20 mM 3-AT. Then extracts were prepared and evaluated by blotting in parallel to the experimental samples. B. Increased levels of phosphorylated eIF2α in *S. pombe trm8Δ* mutants at 38.5˚C were associated with increased expression of *lys4*+ and *aro8*+ mRNAs. Bulk RNA was prepared from the growth done for S17A Fig, and levels of *aro8*+ (SPAC56E4.03) and *lys4*+ mRNAs were quantified relative to *act1*+, using RT-qPCR, as in Fig 5A.

(S18 Fig) A. Analysis of charging levels of tY(GUA) or tP(AGG) in *S. pombe trm8Δ* mutants at 38.5˚C. Strains were grown in YES media at 30˚C and shifted to 38.5˚C, and samples were harvested after 8 hours. Then bulk RNA was isolated and resolved by denaturing PAGE under acidic conditions (to preserve tRNA charging), transferred, and then analyzed by hybridization as described in Materials and Methods. Control samples (WT and *S. pombe trm8Δ* mutants) were treated with 1 mM EDTA and 0.1 M Tris-HCl (pH 9.0) for 30 min at 37˚C to de-acylate the tRNA. b, base treated bulk RNA; Upper arrows, charged tRNA species; lower arrows with dashed lines, uncharged tRNA species. B. Quantification of tY(GUA) or tP(AGG) charging and tRNA levels. The percent charging was calculated as the ratio of aminoacylated species to
the total for each tRNA. Relative levels of tP(AGG) and tY(GUA) were quantified as in Fig 2B, relative to the non-Trm8 substrate tL(UAA).

S19 Fig. A. Northern analysis of pre-tY(GUA) levels in S. pombe WT, trm8Δ, and trm8Δ gcna-1 mutants. The northern blot shown in Fig 5B was continued to analyze levels of pre-tY (GUA) species in WT and trm8Δ, and trm8Δ gcna-1 mutants at different temperatures, using appropriate gene-specific probes (S5 Table) for the introns of the different tY(GUA) genes [59]. Cartoons at the right indicate exons, heavy bars; 5’ leaders, 3’ trailers, and introns, light bars. The primary pre-tY(GUA) transcript has 5’ leader, 3’ trailer, and intron, and the end-matured pre-tY(GUA) has only the intron. B. Quantification of pre-tY(GUA) transcript levels in S. pombe trm8Δ, and trm8Δ gcna-1 mutants, from northern in S19A Fig. The primary pre-tY(GUA) transcript levels were normalized to levels of tG(GCC).

S20 Fig. Deletion of MET22 restored growth of S. cerevisiae trm8Δ trm4Δ gcna2Δ mutants at elevated temperatures. Strains were grown overnight in YPD media 28˚C and analyzed for growth on YPD plates.

S21 Fig. A. Deletion of GCN2 exacerbated the temperature sensitivity of S. cerevisiae trm8Δ and trm1Δ mutants in SDC media. Strains were grown overnight in YPD media at 28˚C and analyzed for growth on SDC plates. B. Deletion of GCN2 exacerbated the temperature sensitivity of S. cerevisiae tan1Δ and tan1Δ trm44Δ mutants in SDC media. Strains were grown overnight in YPD media 28˚C and analyzed for growth on SDC plates.

S22 Fig. A. Northern analysis of pre-tV(AAC) levels in WT, S. cerevisiae trm8Δ trm4Δ, and trm8Δ trm4Δ gcna1Δ cells after shift from 28˚C to 32˚C. Bulk RNA from the growth done for Fig 6B and 6C was used for the northern analysis. B. Quantification of the levels of the primary pre-tV(AAC) transcript. pre-tV(AAC) levels were determined by hybridization with oligomer TDZ 415, specific for seven of the fourteen pre-tV(AAC) species, and then quantification of the upper band, corresponding to the primary transcript, with 5’ leader and 3’ trailer. Levels were normalized to 5S rRNA.

S23 Fig. A. Analysis of tRNA charging levels in S. cerevisiae trm8Δ trm4Δ mutants after shift to 32˚C. Cell pellets from the growth for Fig 6B were used to isolate acidic RNA and analyzed by acidic northern as described in S18 Fig. B. Quantification of tRNA charging and tRNA levels. The percent aminoacylation of tV(AAC) and tK(UUU) was calculated as described in S18 Fig, and relative tRNA levels were quantified as in Fig 6C.

S1 Table. Variable loop sequences and the folding free energies of the acceptor stem/Tstem loop of predicted Trm8 substrate tRNAs of S. pombe trm8Δ mutants.

S2 Table. GAAC mutations identified in S. pombe trm8Δ suppressors.

S3 Table. S. cerevisiae strains used in this study.
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