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The Role of tRNA Modification Systems in the Cellular Stress Response

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The Role of tRNA Modification Systems in the Cellular Stress Response

By

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Abstract

Background

Transfer RNA(tRNA) is a small chain of nucleotides that participates in protein synthesis by pairing its anticodon with an mRNA codon and transferring an amino acid to a growing polypeptide chain. tRNA methyltransferases are a group of enzymes that can modify nucleosides in or around the anticodon, as well as at other parts of the tRNA. Recently, some of these modifications have been reported to enhance the translation of proteins that help the cell respond to and/or repair DNA damage. We hypothesize that the modifications catalyzed by some of the tRNA methyltransferases (Trms) stabilize the interaction between the mRNA codon and the tRNA anticodon thus enhancing the translation of transcripts with specific codon usage patterns. This enhanced translation may increase the levels of certain proteins necessary for the cell to respond to stress. Furthermore, we predict that their activity will dictate cellular responses to environmental carcinogens and chemotherapeutics.

Results

We exposed yeast gene deletion strains to carcinogens that cause cellular stress such as DNA damage, protein stress, and oxidative stress. Thus far, the effects of methyl methanesulfonate, tunicamycin, paramomycin, bleomycin, hygromycin, rapamycin, and hydrogen peroxide have been tested on 13 *trm* knockout strains. The results suggest that three of the tRNA methyltransferases may have a larger role in the stress response than the other ten trms. In particular, those tRNA methyltransferases that act to modify tRNA at the anticodon, or in close proximity to the anticodon, are the most important tRNA methyltransferases involved in

stress responses. Trm4 and Trm5 seemed to be important for response to acute and chronic oxidative stress and this finding was further studied using Western Blots to measure the protein levels of these Trms after oxidative stress. Overall, the results suggest that different tRNA methyltransferases are involved in response to different kinds of stress. Trm4 is important in the oxidative stress response, Trm9 has a part in the response to double strand breaks, and Trm5 has a role in response to both these types of damage.

Conclusion

Cells have a variety of methods for responding to stress and damage. The activation of repair genes and, in turn, various enzymes is one such method. The tRNA methyltransferases are a group of these enzymes that may be activated in response to stress. This response is used to modify tRNAs and enhance translation of damage response transcripts. The mutants *trm9* and *trm5* were the most sensitive to agents tested in this experiment that cause DNA damage and translation inhibition, suggesting that they play an important role in the cellular damage response. *trm4* and *trm5* showed sensitivity to oxidative stress under acute and chronic exposure conditions. When the levels of these proteins were measured after acute exposure, however, the protein levels of the treated samples did not change. This needs to be further studied to understand exactly how these Trms help the yeast respond to oxidative stress. This Since yeast RNA is very similar to that of humans and many tRNA methyltransferase genes in yeast have homologues in humans, this study will ultimately help us understand how tRNA modification systems help human cells respond to stress.

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Background

Human beings are exposed to various carcinogens in the environment everyday. Many of these carcinogens, such as cigarette smoke, UV radiation, and toxic chemicals, including lead, benzene, mercury etc. cause DNA damage (Halazonetis et al. 2008). As a result, mutations in the DNA can occur. If a mutation is in a gene that inhibits tumors or controls cell proliferation, cancer can develop. Accumulation of cells with mutations is an early step in the development of tumors (Halazonetis et al. 2008). To prevent or fix damage, cells have developed many methods of responding to stress including cell cycle arrest, senescence, or apoptosis (Wahl and Carr, 2002; Friedberg et al. 1995).

This study focuses on transfer ribonucleic acids (tRNA) and systems that modify tRNA as a possible source of response to cellular stress. tRNA is a very small nucleic acid, only about 74 to 90 nucleotides in length. Its structure is in the form of a cloverleaf, with the loop at the bottom containing the anticodon. The anticodon is a sequence of three bases that pairs with the three bases of the codon on messenger RNA so translation, i.e. the synthesis of a protein, can occur (Quigley and Rich, 1976). A chain of amino acids is formed as each tRNA anticodon pairs with its corresponding codon on the mRNA. Hence, the proper protein is formed as the tRNA follows the rules of the genetic code in pairing with the mRNA (Nelson and Cox, 2005).

Ribonucleic acids are produced through the process of transcription, forming RNA from the information stored in the sequence of bases in the DNA. It has been shown that although a gene may become transcriptionally active after exposure to a mutagen, this does not indicate whether or not that gene plays a part in recovery. Instead, multiprotein networks have been found that are important in various damage recovery responses (Begley and Samson, 2004).

Since tRNA is a major participant in protein synthesis, it follows that tRNA has a potential role in the cell's damage response.

The modifications that occur on the tRNA help promote stablility and translational efficency. Methylation reactions account for the majority of these posttranscriptional modifications (Ünal et al. 2004). tRNA methyltransferases (Trms) are enzymes that modify tRNA along the entire length of the tRNA including in or around the anticodon. Since modification systems may be important for stress signaling, Trms have a potential and perhaps crucial role in enhacing the synthesis of proteins that participate in the damage response. If a gene that codes for a specific tRNA methyltransferase is absent the cell cannot perform the functions of the missing Trm. Hence, the modification that the Trm catalyzes does not occur and the cell may not be able to respond to damage.

Saccharomyces cerevisiae (budding yeast) is the simplest eukaryotic organism to use to study posttranscriptional modifications (Drubin et al. 2005). In addition, there are around 150 DNA repair and cell cycle checkpoint proteins in humans that help repair DNA damage, and most of them have functional homologues in *S. cerevisiae*. Thus, *S. cervisiae* is a very good model organism for studying the cell's response to DNA damage and inferring how human cells respond to damage. *S. cervisiae* has 17 tRNA methyltransferase genes that modify tRNA. Thirteen of these genes were analyzied in this thesis.

Trm9 is one of the tRNA methyltransferases that has been studied extensively. Trm9 is responsible for the methylation that catalyzes the final step in the formation of 5-methylcarbonylmethyluridine (mcm^{5}U) in tRNA^{Arg3} and 5-methylcarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) in tRNA^{Glu} (Kalhor and Clarke et al. 2003). It enhances the translation of some transcripts with arginine and glutamic acid codons. This suggests a translational role for Trm9

(Begley et al. 2007). A previous study of the *S. cervisiae* tRNA methyltransferase Trm9 has identified this Trm's role in translational enhancement of DNA damage response proteins (Begley et al. 2007). Trm9 has also been shown to be a component of the toxin-target effector Elongator pathway in yeast. This protein complex functions in transcription, exocytosis, and tRNA modification. The toxin zymocin will target modified tRNA and eventually, mRNA translation. Cells where Trm9 is absent are resistant to zymocin because they lack Trm9's modification of U34 (wobble uridine) base. This methylation may help tRNA recognize zymocin (Jablonowski et al. 2006).

Other studies, as well as this one, have shown that when Trm9 is absent in a yeast cell, the cell is less viable than the wildtype after exposure to methyl methanesulfonate (MMS) (Begley et al. 2004). MMS is a damaging agent that methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine, which stalls replication forks and results in DNA double strand breaks. Since mutant *trm9* cells are sensitive to MMS, Trm9's role in translation may be important in the cellular response to double strand breaks (Lundin et al. 2005). Two mechanisms exist to repair DSBs: non-homologous end joining (NHEJ) and recombination repair (also known as template-assisted repair or homologous recombination repair) (Hanway et al. 2002; Paques and Haber, 1999). Trms may have a part in modifying tRNA that assist in the translation of proteins that function in these types of damage repair, as well as other damage responses.

Another type of cellular stress, oxidative stress, has been shown to damage mammalian and *Escherichia coli* cells by causing DNA strand breaks, as well as aldehydic DNA lesions (ADLs) (Pedroso et al. 2008). In this study, hydrogen peroxide was used to induce oxidative stress in the yeast cells. Hydrogen peroxide is able to penetrate biological membranes and generate reactive oxygen species (ROS) in the cell. ROS react with many of the cell's

biomolecules starting a free radical formation chain reaction. This chain reaction cannot stop until a radical reacts with another free radical or a primary antioxidant. In mammals, Thioredoxin reductase (TrxR) combined with thioredoxin (Trx) is a oxidoreductase system with antioxidant and redox regulatory roles (Mustacich and Powis, 1970). Another form of defense against oxidative stress is the tripeptide glutathione. Under oxidative stress glutathione is oxidized and then reduced by glutathione reductase, producing $NADP^+$ and reduced glutathione (Muller et al. 2007). We hypothesize that tRNA methyltrasferases may catalyze modifications to the tRNA that help enhance the level of these oxidative stress response proteins, as well as others.

By treating the knockout tRNA methyltransferase strains with hydrogen peroxide, along with several other damaging agents, we can determine if specific Trms have a role in the DNA damage response. The five agents that were tested in addition to methyl methanesulfonate and hyrdrogen peroxide were all antibiotics. These agents were hygromycin, paromomycin, tunicamycin, bleomycin, and rapamycin. The agents' uses and affect on yeast cells are listed in Table 1.

Results

Agents Tested

Seven agents were tested on thirteen Trm gene deletion strains. These agents are known to cause various types of damage to yeast cells. Methyl methane sulfonate and bleomycin cause cellular damage that leads to double strand breaks. Hydrogen peroxide causes oxidative damage. Hygromycin blocks RNA-protein interactions. Paramomycin causes amino acid misincorporation. Tunicamycin effects the synthesis of a glycoprotein necessary for bud emergence in yeast. Finally, Rapamycin arrests the cell cycle in G1. All of these agents have either a direct or indirect effect on protein synthesis. Most of these agents are used in chemotherapeutics or for other medical purposes.

Sensitive yeast strains, as compared to the wildtype, were found for each agent. These strains included *trm4, trm5, trm9,* and *trm13*. The other 9 strains did not show sensitivity to any of the agents tested.

Mutants Sensitive to MMS

Methyl methane sulfonate (MMS) was the first agent tested. MMS methylates DNA at N^7-deoxyguanine and N^3-deoxyadenine. This stalls replication forks causing DNA double-strand breaks (Lundin et al. 2005). As stated previously, *trm9* is known to be sensitive to MMS exposure (Begley et al. 2007). This experiment confirmed these previous results. The *trm4* mutant also showed less viability than the wildtype when grown in YPD media containing MMS, indicating that these Trms may have a role in responding to double-strand breaks.

Mutants Sensitive to Bleomycin

Bleomycin is an antitumor antibiotic that causes DNA double strand breaks (Claussen and Long, 1999). It is used medically to treat lymphomas, squamous cell carcinomas, and testicular carcinomas (Martindale et al. 2007). *trm5* and *trm9* showed inhibited growth when exposed to bleomycin (Figure 2). Since *trm9* was sensitive to MMS, which also causes double-strand breaks, Trm9's role seems to be important in responding to DNA damage in the form of double-strand breaks. *trm5* was not available when MMS was tested on the other mutants.

Mutants Sensitive to Aminoglycoside Antibiotics

Two aminoglycoside antibiotics were tested on all the Trm knockout strains. Aminoglycoside antibiotics are used to treat bacterial infection because they bind to the tRNA decoding A site in both gram-positive and gram-negative bacteria, thus inhibiting bacterial protein formation (Davies and Wright, 1997). Hygromycin is an aminoglycoside antibiotic that inhibits protein synthesis through a dual effect on mRNA translation (Cabanas et al. 1978). It has also been shown to inhibit spontaneous reverse translocation of tRNAs and mRNA on the ribosome in vitro (Borovinskaya et al. 2008). It is used in gene transfer experiments as a selection antibiotic, since the discovery of hygromycin-resistance genes (Rao et al. 1983). Growth of *trm5* and *trm9* cells were inhibited, once again, when exposed to hygromycin. *trm4* seemed to show some resistance to hygromcin (Figure 3).

Paromomycin was the second aminoglycoside antibiotic tested. This agent blocks translation by promoting amino acid misincorporation (Fan-Minogue and Bedwell et al. 2007). It is used medically to treat infections in the intestines, as well as complications of liver disease

(Vicens and Westhof et al. 2001). *trm5* was the only strain that showed sensitivity to paromomycin (Figure 4).

Mutants Sensitive to Tunicamycin

Tunicamycin, is an antibiotic that inhibits the enzyme GlcNAc phosphotransferase (GPT), thus, blocking transfer of N-actelyglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate in the first step of glycoprotein synthesis (Takatsuki et al. 1971). Tunicamycin affected the growth of *trm13* (Figure 5). This was the only agent to which *trm13* was sensitive. Trm13 is the only tRNA methyltransferase that modifies tRNA at position 4 (Wilkinson et al. 2007). The specific role of Trm13 needs to be further studied to understand if and why this tRNA methyltransferase may be necessary in response to a deficiency in glycoproteins.

Mutants Sensitive to Rapamycin

Another agent tested, rapamycin, caused sensitivity in *trm4* and *trm5* (Figure 6). This drug is an immunosuppressive antibiotic that inhibits the growth and function of certain T and B cells of the immune system involved in the body's rejection of foreign tissues and organs (Pritchard et al. 2005). Its action is to form a rapamycin-FKBP12 (FK-binding protein 12) complex, which inhibits the mammalian target of rapamycin (mTOR) pathway, arresting the cell in the G1 phase (Chan et al. 2004). In yeast, rapamycin inhibits the protein kinase activity of Tor inhibiting cell cycle progression. This mimics the cellular stress caused by amino acid starvation. In cancer treatment, combination therapy of doxorubicin and rapamycin has been shown to cause AKT-positive lymphomas to go into remission in mice. AKT (or protein kinase

B), which has been implicated in many types of cancer, promotes cell survival in lymphomas and prevents the cytotoxic effects of chemotherapy drugs like doxorubicin. Rapamycin can be used to block AKT signaling and cause lymphoma cells to lose their resistance (Sun et al. 2005).

Mutants Sensitive to Hydrogen Peroxide

The final agent, hydrogen peroxide, causes oxidative stress in the cell. Hydrogen peroxide is a by-product of mitochondrial respiration, gives rise to highly reactive -OH radicals, and thus causes endogenous damage to the cell. The effects of hydrogen peroxide on fission yeast have been tested and reveal a stress-activated, mitogen-activated protein kinase pathway responsible in regulating the response (Quinn et al. 2002). *trm4* and *trm5*, again, showed sensitivity to hydrogen peroxide (Figure 7). Hydrogen peroxide and rapamycin have both been shown to effect protein kinase pathways in cells. Since these two *trms* were the only ones sensitive to both hydrogen peroxide and rapamycin, Trm4 and Trm5 may play a role in the activation of protein kinase.

Trm4 is responsible for the methylation of cytosine to methyl-5-Cytosine at several positions on the tRNA. It has been shown in studies that *trm8 trm4* double mutants reveal a distinct rapid tRNA decay (RTD) pathway that degrades preexisting mature tRNA$^{Val(AAC)}$ lacking the corresponding tRNA modifications (Alexandrov et al. 2006). Trm5 is responsible for modifying guanosine to form methyl-1-guanosine through methylation of the tRNA at the position 37, 3'-adjacent to the anticodon. This modification helps prevents tRNA frameshifting, thus assuring correct codon-anticodon pairings.

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Acute Exposure to Agents

All of the previously mentioned damaging agents were tested on the Trm knockouts through chronic exposure. This chronic exposure was over a period of 48 hours. To further test the effect of oxidative stress on *trm4* and *trm5*, acute exposure experiments were performed on these *trms*. This was done through the use of killing curves. The killing curve method involved exposure to hydrogen peroxide over one hour time frame. This being the case, the amount of hydrogen peroxide used for each method was adjusted. In chronic exposure 4 mM and 4.5 mM hydrogen peroxide were used. In the killing curves, 0.5 mM, 2 mM, and 8 mM were used to show increased killing. When exposed to 4.5 mM hydrogen peroxide for 48 hours, four spots of the dilution series grew, as compared to seven spots for the wildtype (Figure 7). In the killing curves, 8 mM of hyrdrogen peroxide caused nearly 100 % killing of the cells (Figures 8 and 9).

The killing curves allowed us to calculate the average percentage of sensitivity of *trm4* and *trm5* as compared to the wildtype, BY4741. At a concentration of 8 mM hydrogen peroxide, *trm4* showed almost 100 % killing, while BY4741 showed about 99% killing. At 8 mM hydrogen peroxide, *trm5* demonstrated about 95% killing, while the wildtype demonstrated about 85% killing. This result shows a little more disparity in the growth between the mutant and the wildtype. The sensitivity of yeast to acute exposure to hydrogen peroxide seems to be high whether it is a wildtype strain or a mutant strain. However, as Figures 8 and 9 show, the mutants do show more sensitivity than the wildtype over varying concentrations of hydrogen peroxide under acute exposure conditions.

Western Blots

Western Blot experiments were performed to discover whether the levels of tRNA methyltransferase proteins in the cell actually do increase or decrease after exposure to damaging agents. The only Trm and agent tested thus far was Trm4 after exposure to hydrogen peroxide. YEF3 was used as a gene that's expression is known to be effected by exposure to hydrogen peroxide. After being treated with hydrogen peroxide for one hour, the levels of untreated Trm4 and treated Trm4 were about the same (Figure 10). However, after exposure to hydrogen peroxide for a half hour, the level of untreated Trm4 was higher compared to the level of treated Trm4 (figure 11). This result does not follow the prediction that the levels of tRNA methyltransferases increase after exposure to damaging agents. Further experiments must be performed to understand why the level of Trm4 decreased after exposure ot hydrogen peroxide

Discussion

In this study, we have shown that a lack of certain tRNA methytransferase genes inhibits growth of yeast after exposure to cellular stress and damage. Some strains showed no sensitivity to any of the agents meaning that they are not necessary for response to the type of damage they were exposed to, they may not be involved in the damage response at all, or they perform modifications that are in redundant to modifications performed by other Trms. Based on the results of these experiments, Trm4, Trm5, and Trm9 seem to have some role in the cell's response to stress, particularly DNA damage, such as double strand breaks and oxidative stress. Presumably, these Trms have similar roles in humans compared to their roles in yeast. The

modifications performed by these Trms may enhance translation of repair proteins that help the cell respond to damage.

One common and debilitating type of damage to cells that was studied in this experiment is DNA double-strand breaks (Khanna and Jackson, 2001). Trm9 has been shown in this experiment, through the mutant's sensitivity to methyl methanesulfonate and bleomycin, to have a role in response to double-strand breaks. The modification performed by Trm9 (mcm^5U) occurs at the anticodon of the tRNA. This modification may enhance translation of proteins necessary for viability after double strand breaks. Proteins of the *RAD52* epistasis group of budding yeast are important for SDSA (synthesis-dependent strand annealing). It has been suggested that among this group, RAD51, RAD52, and RAD54 genes encode the central components of the recombinational repair mechanism (Muris et al. 1994). Trm9 modifications may help the cell to more efficiently translate these proteins.

trm5 was also sensitive to bleomycin. Trm5 modifies the nucleoside 1-methylguanosine (m(1)G37), which prevents frameshifting, and thus the formation of an incorrect or nonfunctional protein. It works at position 37, adjacent to the anticodon. Acting close to the anticodon suggests, just as it does with Trm9, that Trm5 is part of the role in increased translation of damage response proteins. Although *trm5* was not available at the time MMS was tested in this experiment, since exposure to MMS and bleomycin result in DNA double strand breaks, it may be assumed that *trm5* would also be sensitive to MMS. This being the case, the modification catalyzed by Trm5 most likely plays a role in response to double strand breaks. The exact actions of Trm5 and Trm9 in the cell after double strand breaks need to be determined to understand exactly how these Trms are involved in this cellular stress response.

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Another agent that *trm5* and *trm9* were both sensitive to was hygromycin. Hygromycin inhibits protein synthesis through a dual effect on mRNA translation. So, although it does not cause direct damage to the DNA, protein synthesis is affected just as it is when double strand breaks occur. Since Trm5 and Trm9 seem to be important in response to double strand breaks, perhaps the bases they modify enhance translation of transcripts needed to respond to a variety of translational inhibition.

It has been shown that *trm9* is also sensitive to paromomycin but only at the elevated temperature of 37°C (Kalhor and Clarke et al. 2003). This suggests that the esterification performed by Trm9 may be important in response to heat shock. However, since *trm9* was not shown to be sensitive to paramomycin at 30°C, no conclusions about its role in the heat shock response can be drawn from this experiment. *trm5*, however, was sensitive to exposure to paramomycin. Paramomycin promotes amino acid misincorporation, thus, inhibiting proper protein formation. Trm5 again shows a possible role in the response to translational inhibition.

Tunicamycin, another agent tested, inhibits an enzyme needed in the first step of glycoprotein synthesis. This was the only agent that cause inhibited growth in *trm13*. Trm13 is responsible for the 2'-O-methylation of tRNA at position 4. This modification also only occurs in three tRNAs in yeast: tRNA[His], tRNA[Pro], and tRNA[Gly(GCC)] and it is highly conserved in eukaryotes. Perhaps, these three tRNAs are necessary in the formation of glycoproteins. Hence, when Trm13 is absent, tunicamycin causes glycoprotein synthesis inhibition and the cell cannot recover without the modified base at position 4.

The *trm4* and *trm5* mutants were both sensitive to rapamycin and hydrogen peroxide. Rapamycin, as it has been stated, arrests the cell cycle in the G1 phase in T lymphocytes and in yeast cells (Weisman et al. 1997). The G1 (or Gap 1) phase is the interim between mitosis and

DNA replication, during which time the cell continues to grow. Passage through the four phases of the cell cycle is regulated by a family of cyclins that act as regulatory subunits for cyclin-dependent kinases (cdks) (Bartek and Lukas, 2001). If the cell is arrested in G1, the appropriate cyclins that regulate the progression from G1 to S may be absent. Trm4 modifies methyl-5-cytosine near the anticodon. Trm5 methylates guanosine at position 37 in the anticodon, along with modifying methylguanine near the anticodon. The roles of Trm4 and Trm5 may be involved in modifying tRNAs that aid in the translation of these cyclins or other proteins involved in the formation of these cyclins.

The *trm4* and *trm5* mutants also demonstrated inhibited growth in the presence of hydrogen peroxide. Hydrogen peroxide generates reactive oxygen species in the cell that must be neutralized. If they are not neutralized, they can cause damage to proteins, lipids, and nucleic acids. In a genome-wide analysis of yeast gene deletion strains, 394 strains were found to be sensitive to oxidative stress. A large portion of these strains had deletions in genes that have mitochondrial functions, including protein synthesis, respiration, and mitochondrial genome maintenance. (Tucker and Fields 2004). Perhaps Trm4 and Trm5 modify tRNAs that are involved in mitochondrial functioning.

Previous studies conducted on yeast have shown that active Trm5p in the cytoplasm could be obtained in yeast lacking amino acids 1-33 (Delta1-33), whereas production of a Trm5p active in the mitochondria required these first 33 amino acids. Delta1-33 construct exhibited a significantly lower rate of oxygen consumption, indicating that efficiency or accuracy of mitochondrial protein synthesis is decreased in cells lacking the methylation performed by Trm5. These data suggest that this tRNA modification plays an important role in reading frame

maintenance in mitochondrial protein synthesis (Lee et al. 2007). Since cellular respiration takes place in the mitochondria, this indicates Trm5's role in the cell's reaction to oxidative species.

Trm5 most likely plays a crucial role after damage or stress that effects protein translation given the knockouts sensitivity to five of the agents tested (and would have most likely been sensitive to MMS if tested) that indirectly inhibit translation, as well as directly inhibit translation. Trm5 modifies several bases near the anticodon. In particular, it seems to be involved in response to oxidative stress and double strand breaks. However, all of the damage caused by these agents to which *trm5* was sensitive, including cell cycle arrest, inhibition of RNA-protein interactions, and double strand breaks, affect the synthesis of proteins in the cell. Thus, Trm5 seems to have an extremely important role in enhancing translation of damage response proteins.

Since *trm4* and *trm5* both displayed sensitivity to hydrogen peroxide, and several other yeast genes seem to be implicated in the response to oxidative stress, the effects of oxidative stress on these *trms* were further studied. To do this, killing curves were performed. Further studies need to be done to see what proteins have enhanced translation as a result of the Trms activation in response to oxidative stress and if these proteins are activated more under chronic stress rather than acute. Enhanced translation of proteins such as thioredoxin reductase and glutathione, which are involved in the cell's oxidative stress response, may occur as a result of increased modifications catalyzed by Trm4 and Trm5. These results demonstrate that perhaps Trm4 and Trm5 function in more long term recovery from damage and stress.

In addition, we predicted that the levels of Trm4 and Trm5 will increase after oxidative stress as caused by exposure to hydrogen peroxide. After treatment with 5 mM of hydrogen peroxide for one hour, the protein levels of the treated Trm4 sample compared to the control

were about the same, as demonstarted by a Western Blot. However, after treatment with

hydrogen peroxide over a half-hour period, the level of untreated Trm4 increased as compared to

the level of treated Trm4 (Figure 9). The reason for this after exposure for a half hour as

opposed to an hour needs to be explored. When the killing curve experiments were performed,

an hour of treatment had a killing effect on the *trm4* knockouts. However, perhaps different

conditions affect the increase in the Trm4 protein level. Trm4 may still have increases in its

modifications without actually increasing its own protein level. Perhaps just the presence of

Trm4 helps enhance the level of oxidative damage response proteins. Future studies using

varying concentrations of hydrogen peroxide and varying exposure times can be done to see the

effect of these different parameters on the Trm4 protein levels. Western Blots can also be done

with the other tRNA methyltransferases and using the other damaging agents to see if the protein

levels of the other Trms are affected by various damage and if the protien levels actually increase

after damage as predicted.

Since we identified that Trm4, Trm5, and Trm9 are important for viability and growth

after exposure to certain kinds of cellular damage, future studies can also be done to observe the

proteins that these *TRM* genes activate and determine the functions of these proteins. We can

then understand the effects of expression (or non-expression) of these genes and their role in the

stress response. Since tRNA methyltransferases methylate tRNA, we can study biological

methylation of yeast tRNA *in vivo* by incubating the cells with *S*-adenosyl-L-[*methyl*-3H]

methionine ([3H]AdoMet), the major biological methyl donor. Through this method, methyl-

accepting species such as RNAs, proteins, and small molecules can become radiolabeled and the

fate of the methylated species can be followed biochemically. We can then look for differences

in the methylation spectra between a mutant strain and the wildtype (Kalhor et al. 2008).

Materials & Methods

To observe the role of tRNA methyltransferases in response to stress caused by damaging agents, part of a *Saccharomyces cerevisiae* BY4741 gene deletion library was used (Brachmann et al. 1998). Thirteen strains, each deficient in one Trm gene, as well as the wildtype strain were exposed to seven different damaging agents. The resulting growth of each strain after exposure to a particular agent was used to determine its sensitivity to that agent. Deletion strains that show sensitivity means that the deleted gene is necessary for efficient cellular response to a given stressor.

To begin the experiment, the thirteen *S. cerevisiae* single-gene deletion strains were inoculated overnight in YPD. YPD is a rich media that contains all the nutrients needed for yeast to grow. It is made from yeast extract, peptone, dextrose, and distilled water. The following day, a dilution series of each strain in YPD was made in a 96-well plate. The dilution series were then spotted on plates containing YPD media and varying concentrations of the agent of interest. Varying concentrations ranging from nontoxic to lethal were used to test for the concentration at which most strains would survive but some strains would be sensitive. The strains were also spotted on a control plate of YPD without any damaging agent. The plates were left in 30º for 48 hours to allow the yeast cells to grow. Then, the growth of each strain on the plates containing the damaging agents was compared to the growth of the respective strain on the untreated plate. Those strains, whose growth was significantly less than the other strains in the presence of damaging agent compared to on the untreated plate, as well as the wildtype, were deemed sensitive.

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This method of analysis of the sensitivity of certain yeast genotypes is similar to genomic phenotyping (Begley, Rosenbach, Ideker, and Samson et al. 2002). Through this method one can identify specific genes that exhibit a particular phenotype. In this case, the phenotype was the sensitivity of *S. cerevisiae* strains on exposure to mutagens. Based on their sensitivity when a gene was lacking, we can predict which tRNA methyltransferases influence recovery after exposure.

Further testing of the effect of oxidative stress on the *trm* mutants was done using killing curves. Killing curves involve acute exposure to a toxin as opposed to the previous method, which involved chronic exposure. The mutants *trm4* and *trm5* showed sensitivity to chronic exposure, so their sensitivity to acute exposure was tested. To begin, the strains were inoculated overnight to allow for growth. The next day, the strains were sub-cultured and grown to 5×10^6 cells/mL. Then 3 mL of each strain was put in test tubes and treated with varying concentrations of H_2O_2: 0 mM, 0.5 mM, 2 mM, and 8 mM. The test tubes were then inoculated for one hour. After inoculation, the strains were spread on plain YPD plates, estimating about 200 cells per plate for the cells that were untreated with hydrogen peroxide. This would allow us to count the cells that were treated with hydrogen peroxide and compare that number to the amount of untreated. The plates were left in a temperature of 30^oC to allow the cells to grow for 48 hours. After this time period, the number of cells on each plate was recorded. The percent growth was calculated for each strain at each concentration as compared to the plate containing cells of that strain that had not been treated with any hydrogen peroxide. This method allowed us to see the degree of hydrogen peroxide sensitivity of *trm4* and *trm5* compared to the wildtype.

Finally, Western Blots were performed on Trm4 to observe whether the level of this protein increases after exposure to hydrogen peroxide. A sodium dodecyl sulfate polyacrylamide

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gel (SDS-PAGE) was used to separate the proteins based on protein size (Shapiro et al. 1967).

The strains were Tap-Tagged and Magic Mark XP Western Standard Marker was used as a

marker to identify the protein sizes. TRM4, as well as RNR3 and YEF3, were grown overnight

and then subcultured in 50 mL YPD to grow to 5 x 10^6 cells/mL. The samples were then

separated in two with one half as the untreated or control sample and one half treated with 5 mM

H_2O_2 for. The samples were grown in the inoculator for a half hour and then, in a separate

experiment, for an hour.

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Figure Legends

Figure 1 – Trms grown 48 hours on YPD containing Methylmethane sulfonate

YPD plates containing bleomycin with a dilution series of all the mutant strains spotted on the plates. *trm5* and *trm9* show inhibited growth on this media.

Figure 2 – Trms grown for 48 hours on YPD containing Bleomycin

YPD plates containing bleomycin with a dilution series of all the mutant strains spotted on the plates. *trm5* and *trm9* show inhibited growth on this media.

Figure 3 – Trms grown for 48 hours on YPD containing Hygromycin

YPD plates containing hygromycin with a dilution series of all the mutant strains spotted on the plates. *trm5* and *trm9* show inhibited growth on this media.

Figure 4 – Trms grown for 48 hours on YPD containing Paramomycin

YPD plates containing paramomycin with a dilution series of all the mutant strains spotted on the plates. *trm5* shows inhibited growth on this media. *trm8, trm9, trm10, trm12, trm13, trm44,* and *trm82* showed no sensitivity compared to the wildtype.

Figure 5 – Trms grown for 48 hours on YPD containing Tunicamycin

YPD plates containing tunicamycin with a dilution series of all the mutant strains spotted on the plates. *trm13* shows inhibited growth on this media. *trm1, trm2, trm3, trm4, trm5,* and *trm7* showed no sensitivity compared to the wildtype.

Figure 6 – Trms grown for 48 hours on YPD containing Rapamycin

YPD plates containing rapamycin with a dilution series of all the mutant strains spotted on the plates. *trm4* and *trm5* show inhibited growth on this media. *trm8, trm9, trm10, trm12, trm13, trm44,* and *trm82* showed no sensitivity compared to the wildtype.

Figure 7 – Trms grown for 48 hours on YPD containing hydrogen peroxide

YPD plates containing hydrogen peroxide with a dilution series of all the mutant strains spotted on the plates. *trm4* and *trm5* show inhibited growth on this media. *trm8, trm9, trm10, trm12, trm13, trm44,* and *trm82* showed no sensitivity compared to the wildtype.

Figure 8 – Killing Curve of *trm4Δ*

Percent growth of wildtype strain compared to *trm4* after acute exposure (1 hr) to hydrogen peroxide. *trm4* shows inhibited growth compared to the wildtype.

Figure 9 – Killing Curve *trm5Δ*

Percent growth of wildtype strain compared to *trm5* after acute exposure (1 hr) to hydrogen peroxide. *trm5* shows inhibited growth compared to the wildtype.

Figue 10 – Western Blot of *trm4Δ*

Western Blot of Tap-tagged strains with a Magic XP Western Standard Marker. Samples treated for 1 hour with hydrogen peroxide. Trm4, which is about 70 KD, appears at around 100 KD because of the Tap-tag, which is about 30 KD. The levels of Trm4 protein treated and untreated were very similar.

Figue 11 – Western Blot of *trm4Δ*

Western Blot of Tap-tagged strains with a Magic XP Western Standard Marker. Smaples treated for one half hour with hydrogen peroxide. Trm4, which is about 70 KD, appears at around 100 KD because of the Tap-tag, which is about 30 KD. The levels of Trm4 protein decrease after this hydrogen peroxide exposure.

Tables

Table 1. Damaging Agents Tested and their Effects on Yeast DNA and Translation

Agent	Use	Stress on Cells
Methyl Methane Sulfonate	Chemical mutagen	Double strand breaks
Hydrogen peroxide	Oxidative Therapy	Oxidative stress
Hygromycin	Aminoglycoside antibiotic	Blocks translation of mRNA
Paramomycin	Aminoglycoside antibiotic	Promotes amino acid misincorporation
Tunicamycin	Antibiotic	Inhibits glycoprotein synthesis
Bleomycin	Antitumor antibiotic	Double strand breaks
Rapamycin	Immunosupressive antibiotic	Arrests cell cycle in G1

Table 2. Functions of tRNA methyltransferases

Gene Name	Strain	Substrate Specificity	Mutant Phenotype	Human Homologues
Trm1	YDR120C	modified base N2,N2-dimethylguanosine	viable exhibits growth defect on a non-fermentable carbon source reduced fitness in rich medium (YPD) uncharacterized allele affects a base modification of cytoplasmic and mitochondrial tRNA	Trmt1
Trm2	YKR056W	methylates the uridine residue at position 54 role in tRNA stabilization or maturation endo-exonuclease DNA repair	viable	HTF9C
Trm3	YDL112W	catalyzes the ribose methylation of the guanosine nucleotide at position 18	viable null mutants are defective for Gm18 tRNA ribose methylation	TARBP1
Trm4	YBL024W	methylates cytosine to m5C at several positions similar to Nop2p and human proliferation associated nucleolar protein p120	Viable decreased Ty3 transposition null mutant is viable, sensitive to paromomycin, lacks m5C methylation in total yeast tRNA	NSUN2
Trm5	YHR070W	methylates methylates guanosine at position 37 to help prevent frameshifting	inviable	TRMT5
Trm7	YBR061C	methylates the 2'-O-ribose of nucleotides at positions 32 & 34 of tRNA anticodon loop	viable slow-growth, translation is impaired, sensitive to paromomycin point mutation within the AdoMet-binding domain affects function of enzyme	RRMJ1
Trm8	YDL201W	subunit of a tRNA methyltransferase complex composed of Trm8p and Trm82p that catalyzes 7-methylguanosine modification of tRNA	viable	TRMB
Trm9	YML014W	catalyzes the esterification of modified uridine nucleotides in tRNAs creates 5-methylcarbonylmethyluridine in tRNA(Arg3) and 5-methylcarbonylmethyl-2-thiouridine in tRNA(Glu) role in stress response	viable exhibits growth defect on a fermentable carbon source. decreased drug resistance (rapamycin) normal drug resistance (wortmannin) reduced fitness in rich medium (YPD)	
Trm10	YOL093W	methylates the N-1 position of guanosine in tRNAs	viable	NP_689505.1
Trm12	YML005W	S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family	viable	TRMT12

		required for wybutosine formation in phenylalanine-accepting tRNA		
Trm13	YOL125W	modification of tRNA at position 4	viable	CCD76
Trm44	YPL030W	green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm non-essential gene	viable	NP_689757.1
Trm82	YDR165W	catalyzes 7-methylguanosine modification of tRNA	viable	WDR4

Figure 1.

Untreated   3.0 mM MMS   5.0 mM MMS

Figure 2.

Figure 3.

| | Untreated | 50 µg/mL Hygromycin | 75 µg/mL Hygromycin |

Figure 4.

| | Untreated | 1 mg/mL Paramomycin | 2 mg/mL Paramomycin |

Figure 5.

	Untreated	4.0 mM Tunicamycin	4.5 mM Tunicamycin

trm82Δ
trm44Δ
trm13Δ
trm12Δ
trm10Δ
trm9Δ
trm8Δ
WT

Figure 6.

	Untreated	30 mM Rapamycin	50 M Rapamycin

trm7Δ
trm5Δ
trm4Δ
trm3Δ
trm2Δ
trm1Δ
WT
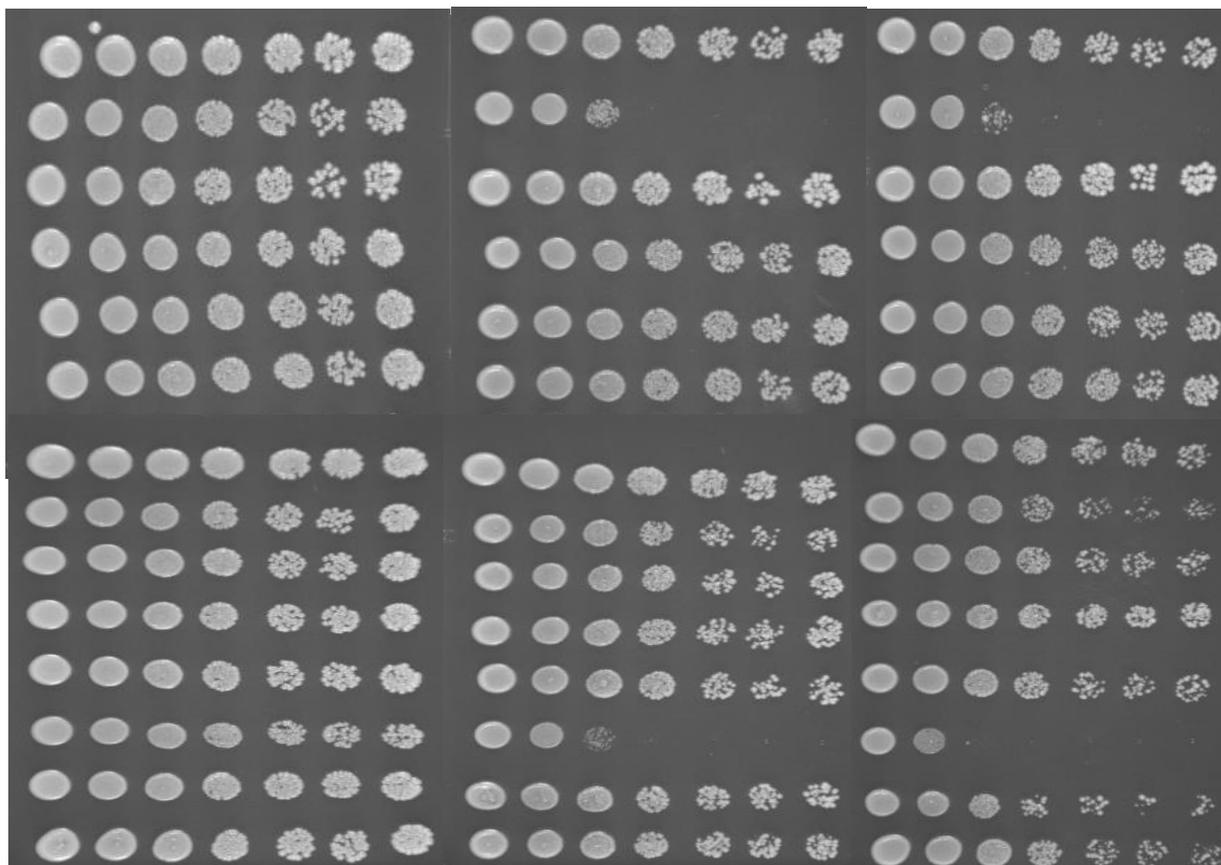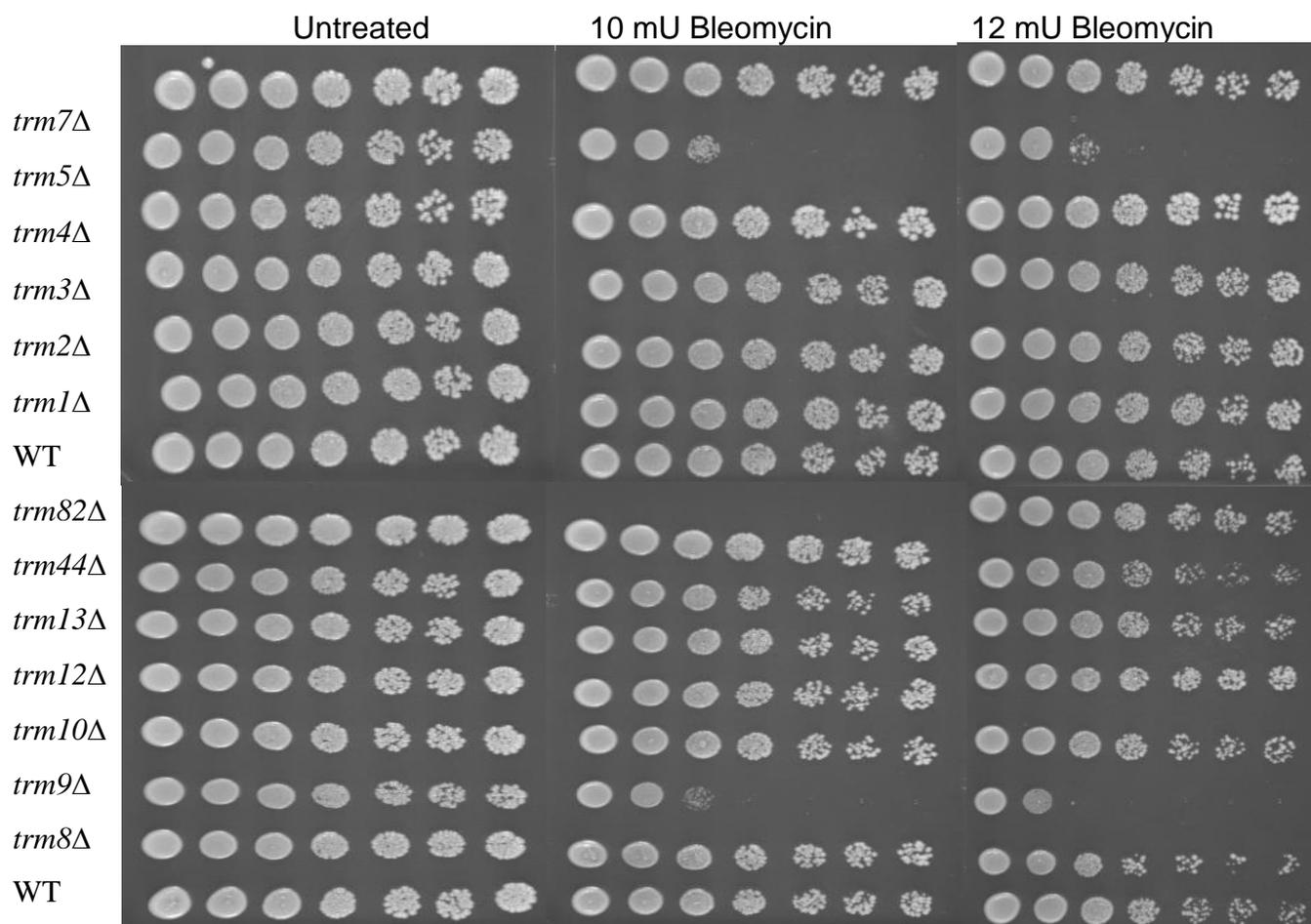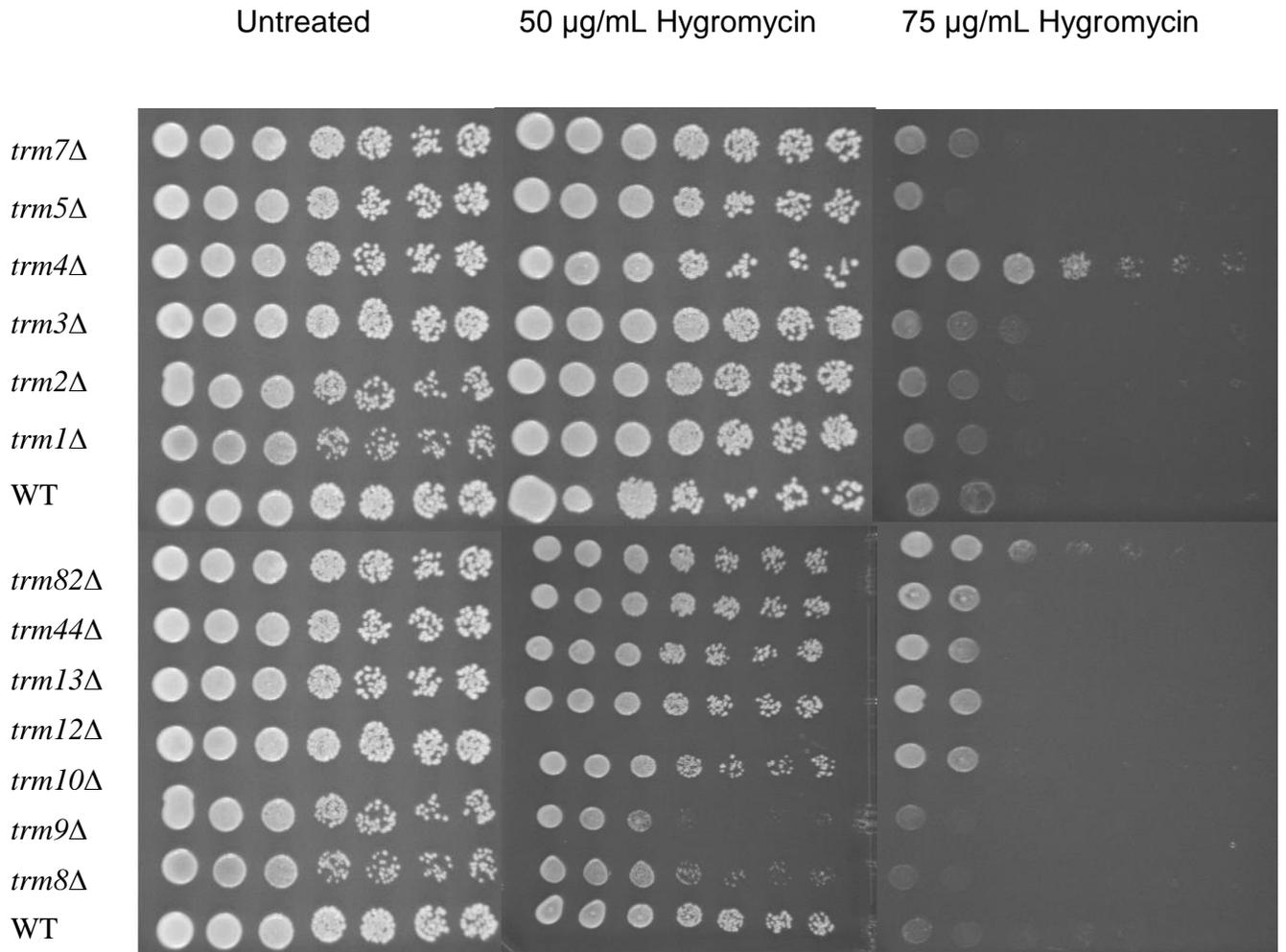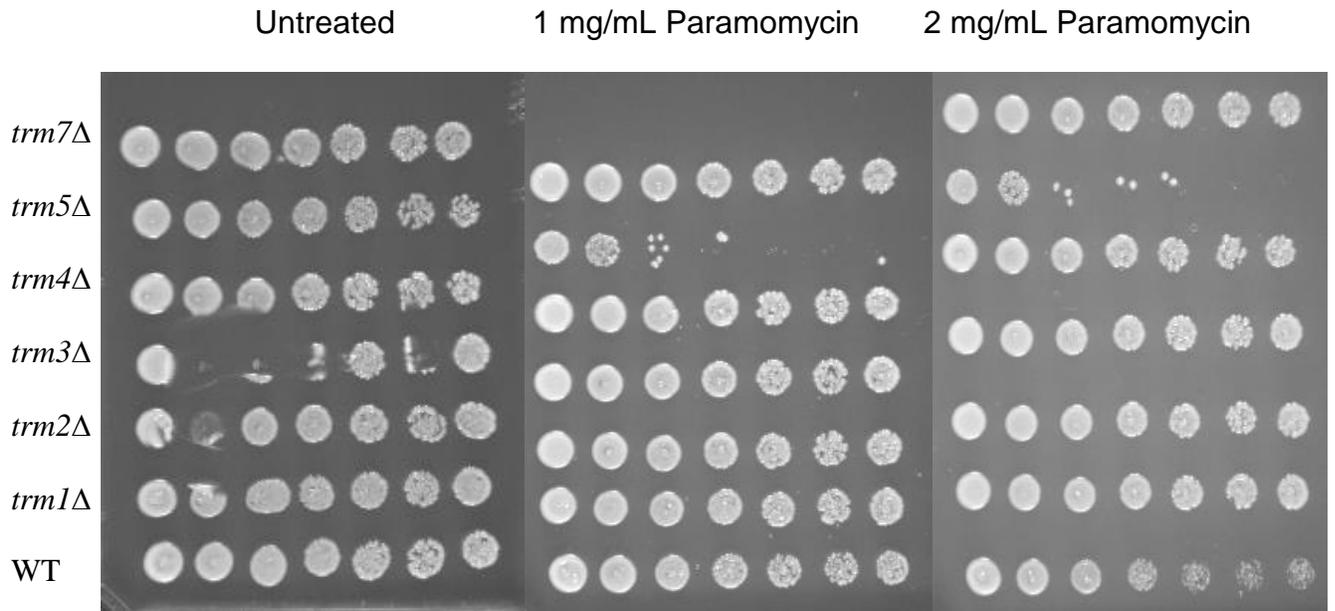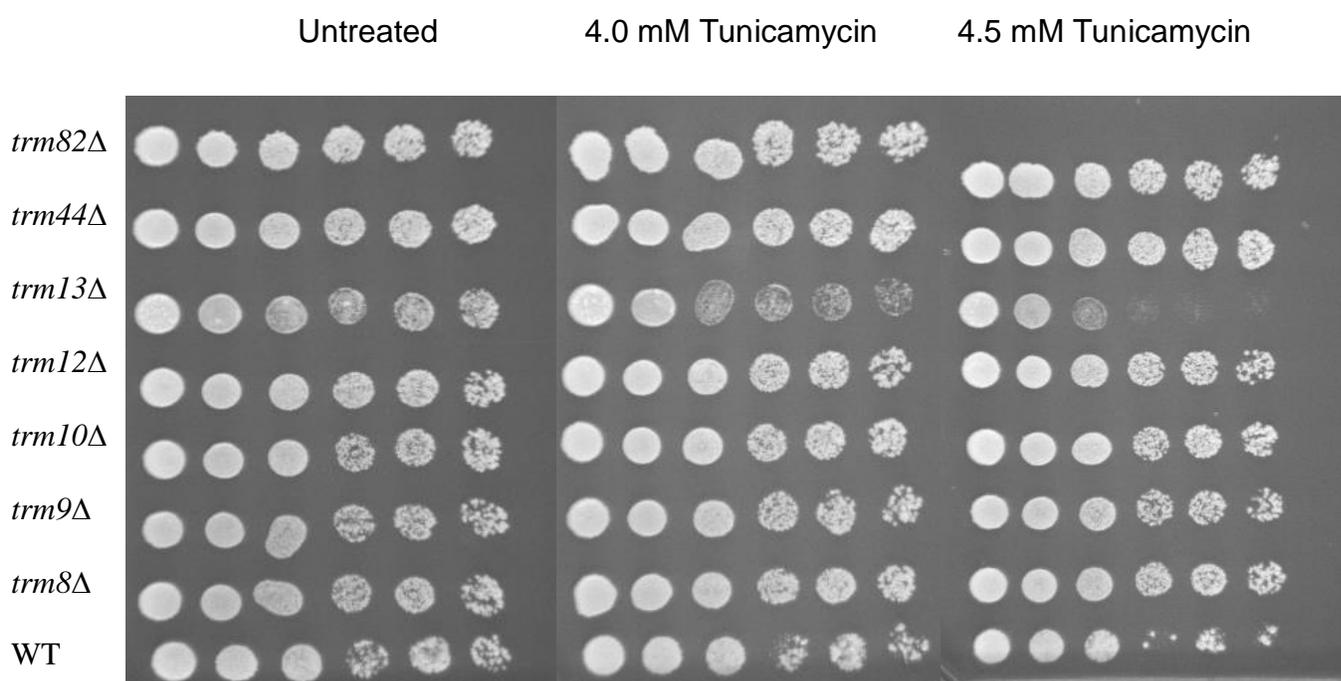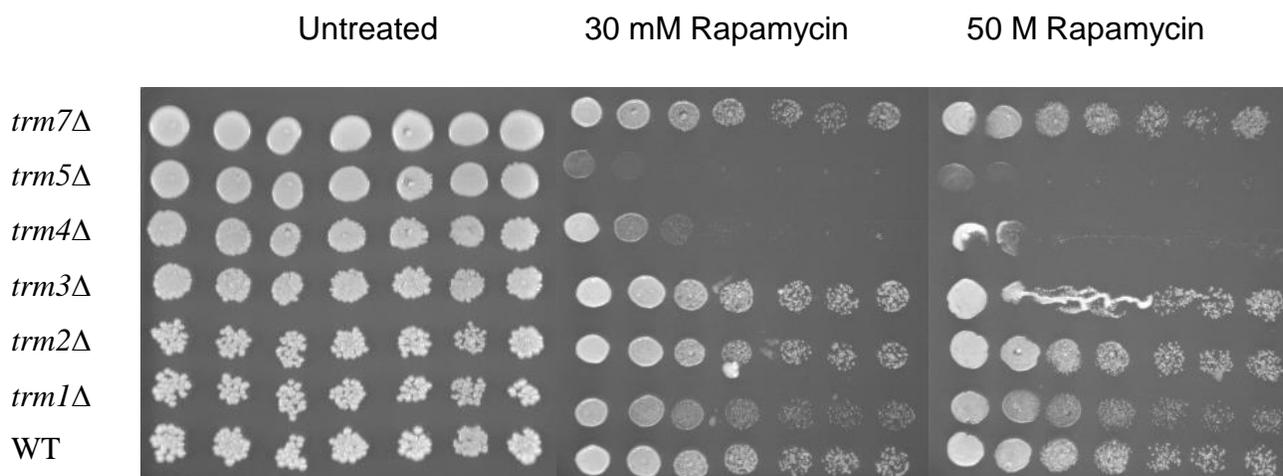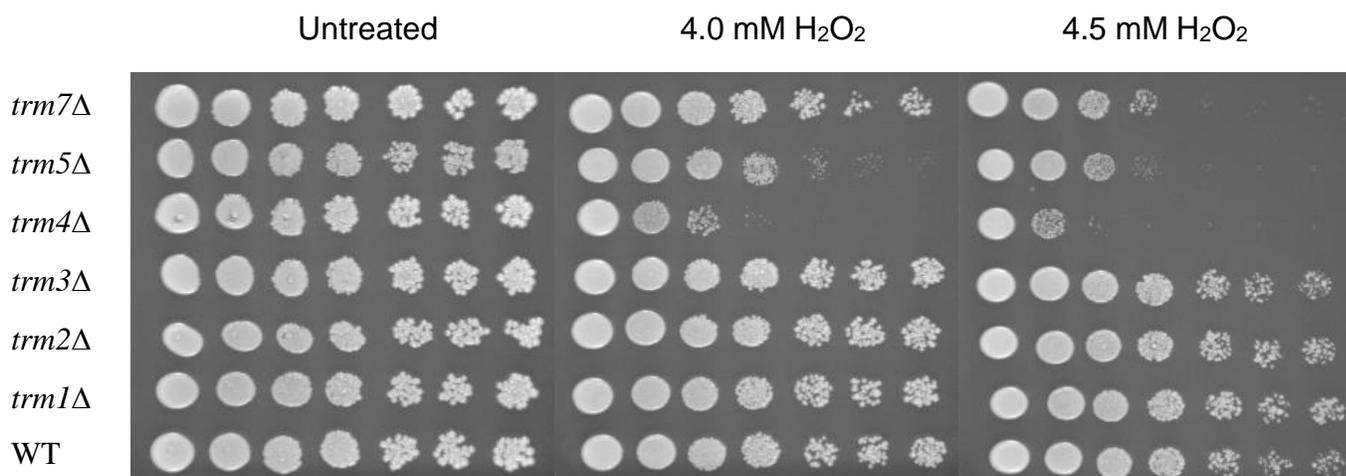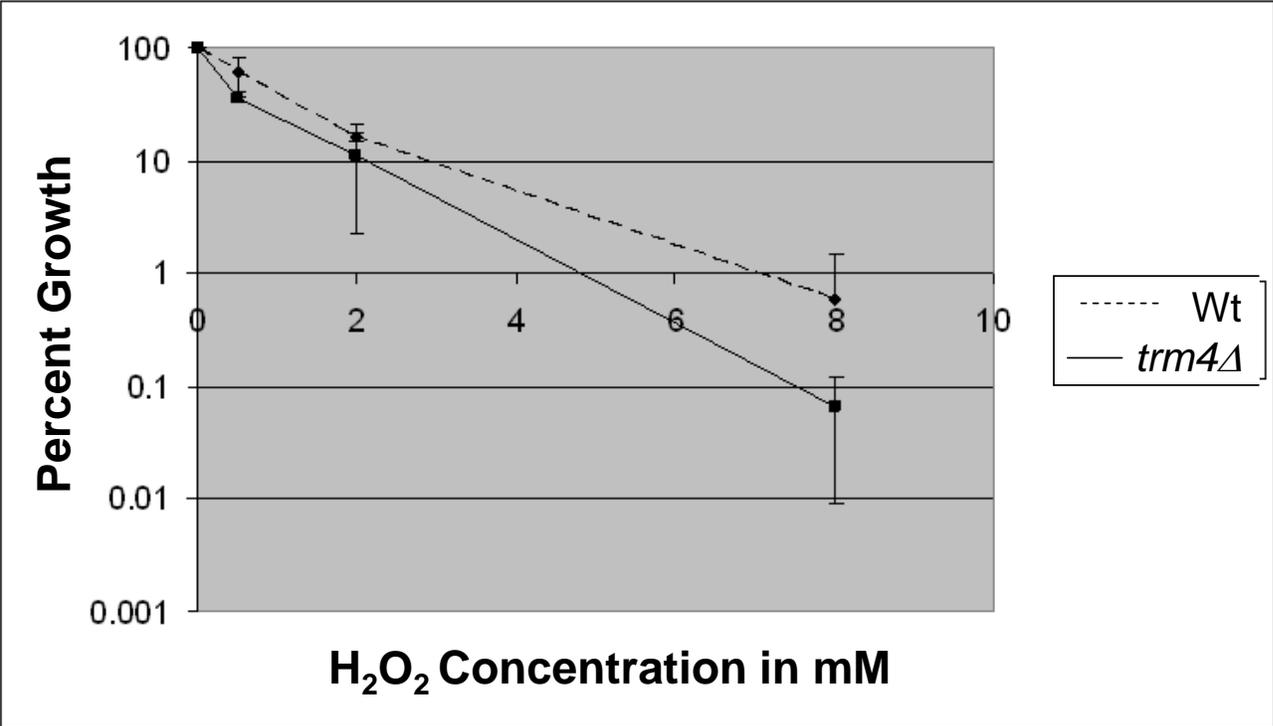
Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 11.