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# Genome-wide analysis of N<sup>1</sup>-methyl-adenosine modification in human tRNAs

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## ABSTRACT

The N<sup>1</sup>-methyl-Adenosine (m<sup>1</sup>A58) modification at the conserved nucleotide 58 in the TΨC loop is present in most eukaryotic tRNAs. In yeast, m<sup>1</sup>A58 modification is essential for viability because it is required for the stability of the initiator-tRNA<sup>Met</sup>. However, m<sup>1</sup>A58 modification is not required for the stability of several other tRNAs in yeast. This differential m<sup>1</sup>A58 response for different tRNA species raises the question of whether some tRNAs are hypomodified at A58 in normal cells, and how hypomodification at A58 may affect the stability and function of tRNA. Here, we apply a genomic approach to determine the presence of m<sup>1</sup>A58 hypomodified tRNAs in human cell lines and show how A58 hypomodification affects stability and involvement of tRNAs in translation. Our microarray-based method detects the presence of m<sup>1</sup>A58 hypomodified tRNA species on the basis of their permissiveness in primer extension. Among five human cell lines examined, approximately one-quarter of all tRNA species are hypomodified in varying amounts, and the pattern of the hypomodified tRNAs is quite similar. In all cases, no hypomodified initiator-tRNA<sup>Met</sup> is detected, consistent with the requirement of this modification in stabilizing this tRNA in human cells. siRNA knockdown of either subunit of the m<sup>1</sup>A58-methyltransferase results in a slow-growth phenotype, and a marked increase in the amount of m<sup>1</sup>A58 hypomodified tRNAs. Most m<sup>1</sup>A58 hypomodified tRNAs can associate with polysomes in varying extents. Our results show a distinct pattern for m<sup>1</sup>A58 hypomodification in human tRNAs, and are consistent with the notion that this modification fine tunes tRNA functions in different contexts.

**Keywords:** N<sup>1</sup>-methyl-adenosine; tRNA; modification; microarray

## INTRODUCTION

Over 100 types of RNA modifications have been identified in thousands of sites in tRNAs, rRNAs, mRNAs, snRNAs, and other RNAs (Bjork 1995; Maxwell and Fournier 1995; Rozenski et al. 1999; Ebhardt et al. 2005; Yu et al. 2005a,b). A human tRNA contains, on average, 13 modifications. A highly conserved modification in eukaryotic tRNA is N<sup>1</sup>-methyl-adenosine at position 58 (m<sup>1</sup>A58) (Fig. 1A). A58 forms a reverse Hoogsteen pair with conserved U54 in the tertiary structure of tRNA. Methylation at the N<sup>1</sup> position of A58 introduces a positive charge at the elbow region of the tRNA tertiary structure without disrupting any hydrogen bonding interactions. This positive charge is thought to stabilize the structure of certain tRNAs on the

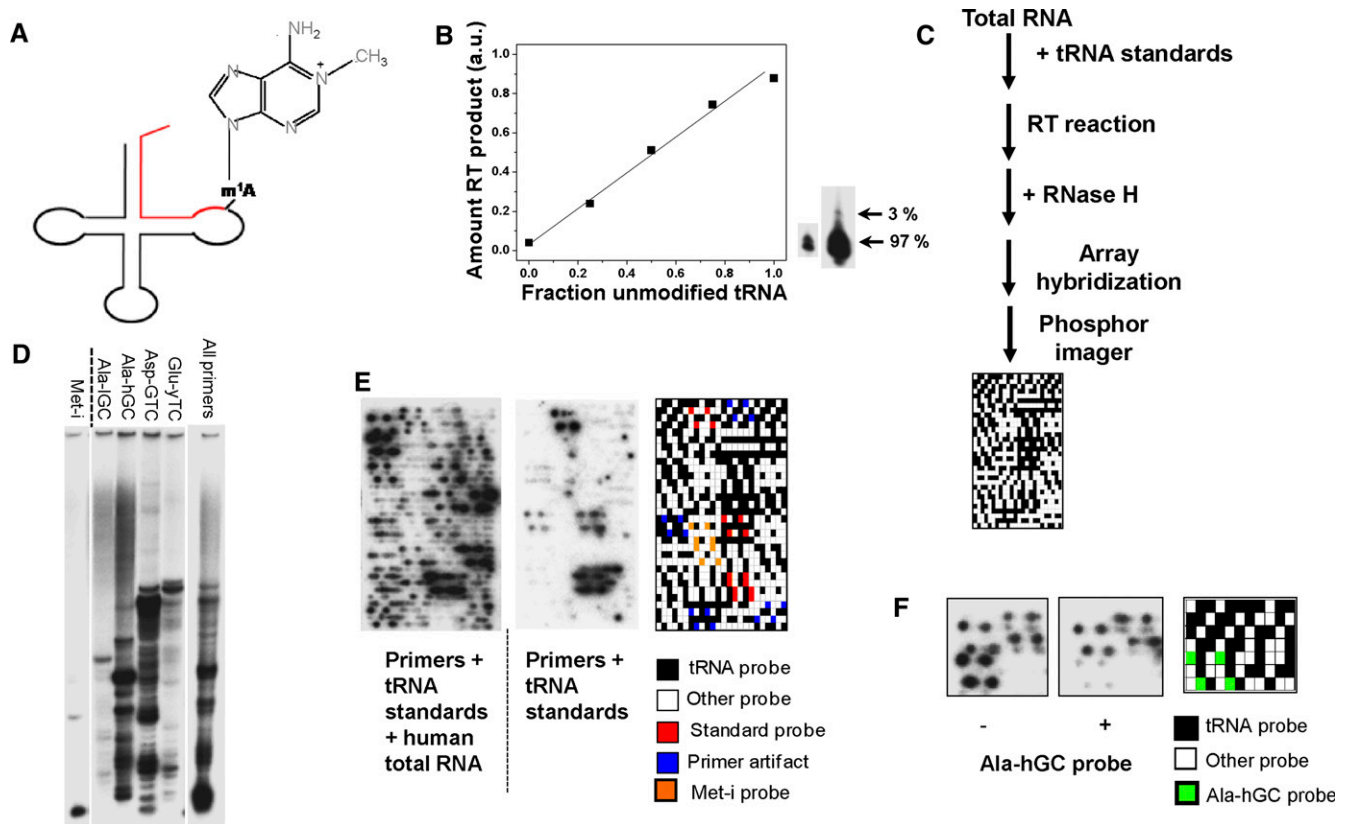
basis of the crystal structure of yeast initiator-tRNA<sup>Met</sup> (Basavappa and Sigler 1991).

In yeast, inactivation of either subunit of the m<sup>1</sup>A58 methyltransferase (TRM6 and TRM61) results in growth arrest and cell death due to the rapid degradation of initiator tRNA<sup>Met</sup> lacking m<sup>1</sup>A58 modification (Anderson et al. 1998, 2000; Kadaba et al. 2004). Mutations of TRM6 and TRM61 cause derepression of GCN4 translation and its target genes because of the reduced level of initiator-tRNA<sup>Met</sup> (Cuesta et al. 1998; Calvo et al. 1999). The role of m<sup>1</sup>A58 in stabilizing the initiator-tRNA<sup>Met</sup> structure has been proposed to derive from its unique A54–A58 interaction (all other tRNAs in yeast have T54–A58 interaction) (Sigler 1975; Schevitz et al. 1979; Basavappa and Sigler 1991). Alternatively, the absence of m<sup>1</sup>A58 leads to the formation of a non-tRNA-like structure for the initiator-tRNA<sup>Met</sup> (Anderson and Droogmans 2005). Both explanations are consistent with the observation that three other tRNAs in yeast, tRNA<sup>Met</sup>, tRNA<sup>Ile</sup><sub>UAU</sub>, and tRNA<sup>Ser</sup><sub>CGA</sub>, were not destabilized by the lack of this modification (Anderson et al. 1998, 2000; Calvo et al. 1999).

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**FIGURE 1.** A microarray method to study  $m^1A$  modifications in human tRNA. (A) Location of  $N^1$ -methyl-A modification in tRNA. The RT primers are complementary to the region in red. (B) The amount of RT product is proportional to the amount of unmodified template tRNA. The primer is complementary to nucleotides 76–59, and all four dNTPs are included. The small amount of hypomodified yeast tRNA<sup>Phe</sup> at  $x = 0$  is confirmed by another primer extension using the primer complementary to nucleotides 76–60 and a mixture of dATP, dTTP, dCTP, and ddGTP. A short exposure of the same lane on the *left* shows the separation of primer and primer stop at  $m^1A58 (+1)$ . (C) Scheme of a microarray experiment for  $m^1A$  modification studies. (D) Detection of RT product using primers for individual human tRNA families. (E) Detection of RT products using a microarray. The array with a total human RNA sample (MCF10A cell line, *left*) shows much more products than the array with primers and standard tRNAs alone (*middle*). The array key is on the *right*. The array results correlates well with the gel analysis. For example, both methods show a very low amount of product for Met-i and a very large amount of product for Ala-hGC. (F) Specificity of the array method is indicated by the specific loss of the Ala-hGC signal when a large amount of the Ala-hGC oligo is added in array hybridization. The array key is on the *right*.

Most mammalian tRNAs contain  $m^1A58$  modification (Anderson and Droogmans 2005). Among the over 30 tRNAs sequenced, only two, rat tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup>, have been reported to be unmodified at position 58 (Kuchino et al. 1981; Chan et al. 1982). The human homolog of the yeast tRNA  $m^1A$  methyltransferase has been identified and it has been shown to have the same two subunit composition as the methyltransferase from yeast (Ozanick et al. 2005).

Very few reports exist, however, on whether the  $m^1A58$  modification is always complete for each tRNA species, or a fraction of some tRNA species may be hypomodified at this position inside cells. In principle, a varying level of  $m^1A58$  modification can be derived from low availability of cosubstrates (e.g., S-adenosyl-methionine used by the methyltransferase), regulated concentration or activity of the  $m^1A58$ -modification enzyme, or an imbalance in the speed of tRNA folding and the modification reaction. Indeed, it has been reported that rat mammary adenocarcinoma has reduced overall  $m^1A58$  levels in tRNA, and this reduction has been

attributed to low methyltransferase levels in these tumors (Salas et al. 1982).

What are the pros and cons for cells to produce hypomodified tRNAs? On the neutral side, some modifications may be crucial only under certain conditions, so that the presence of hypomodified tRNAs is easily tolerated (Salas et al. 1982). On the negative side, hypomodified tRNAs may become dominant-negative inhibitors of tRNA aminoacylation or decoding of mRNA codons (Gustilo et al. 2008). On the positive side, hypomodified tRNAs represent multiple diverse tRNA species that may perform the same function at different efficiencies or may even perform distinct functions. Lack of a specific modification may weaken its interaction with the translation machinery, so that the hypomodified tRNA can be used more efficiently in cellular processes not directly involving ribosomes or elongation factors. For instance, hypomodified tRNAs may be more efficient activators of the GCN2 kinase in eukaryotes (Cuesta et al. 1998; Calvo et al. 1999).

Because hypomodified tRNAs may impact cell biology in many ways, it will be of interest to query which tRNAs are indeed hypomodified in the cell. Traditional methods involve the digestion of tRNA to fragments or mononucleosides, followed by chromatographic or mass spectrometric analysis (Kowalak et al. 1993; Yu et al. 1997; Qiu and McCloskey 1999; Zhao and Yu 2004). Although powerful, these methods generally require purification of individual tRNAs from a mixture of total tRNAs in some way, hence making it difficult to perform at the genomic scale. Furthermore, the presence or absence of certain modifications often impacts the purification procedure, complicating the interpretation for the analysis of hypomodification.

This work investigates m<sup>1</sup>A58 hypomodification in all human tRNAs and initial characterization of the functional significance of hypomodification in individual tRNA species. To simultaneously detect the presence of m<sup>1</sup>A58 hypomodified tRNA species in a total RNA mixture, we first perform primer extension using all primers complementary to the nucleotides 76–59 of all tRNAs. Because reverse transcriptase reads through m<sup>1</sup>A at a very low efficiency (Maden 1990; Maden et al. 1995; Kiss-Laszlo et al. 1996), only m<sup>1</sup>A58 hypomodified tRNAs can be extended at appreciable amounts. The primer extension products are then detected using a microarray method. We found that among the five cell lines examined, approximately one-quarter of human tRNA species contain hypomodified A58, and the pattern and the extent of hypomodified tRNAs are similar. We compare the hypomodified to m<sup>1</sup>A58-modified tRNAs in their relative association with polysome that approximates their involvement in translation. Our results indicate that cellular tRNAs are diverse in their modification status. The naturally present hypomodified tRNAs can have different efficiencies in polysome association, suggesting possible diversification in function.

## RESULTS

### A microarray method to analyze m<sup>1</sup>A58 hypomodification in tRNA

Our method makes use of the extremely low ability of reverse transcriptase (RT) to incorporate a base opposite to the N<sup>1</sup>-methyl-A modified nucleosides, thus leading to a stop during primer extension (Maden 1990; Maden et al. 1995; Kiss-Laszlo et al. 1996). Studies in Moloney murine Leukemia virus as well as HIV virus have shown that m<sup>1</sup>A plays a pivotal role in termination of reverse transcription in these viruses (Gilboa et al. 1979; Burnett and McHenry 1997; Renda et al. 2001). We utilized this inherent property of N<sup>1</sup>-methyl-A modification as the detection signal for hypomodification at A58 in human tRNA molecules.

To validate that reverse transcription can be used to quantitatively compare the extent of m<sup>1</sup>A58 in a tRNA, we performed primer extension using defined mixtures of purified yeast tRNA<sup>Phe</sup>, which contain this modification, and

synthetic full-length yeast tRNA transcript, which is unmodified (Fig. 1B). The primer for tRNA<sup>Phe</sup> was designed to be complementary to the region encompassing the 3' CCA to nucleotide 59 of the tRNA. This way, only tRNAs with unmodified A58 can be extended by RT. The reverse-transcription products range from RT stops at residue 37 where the wybutosine (Y-base) is located to run-off reverse transcripts at the end of tRNA. Using 5' <sup>32</sup>P-labeled primers, all products from RT stops at Y37 to RT run-off were quantified. We found that the amount of product increases linearly from very low ( $x = 0$ ,  $y = 0.04$  when only yeast tRNA<sup>Phe</sup> isolated from the cell was present) to very high ( $x = 1$ ,  $y = 0.88$  when only tRNA<sup>Phe</sup> transcript was present). The small amount of product detected in the RT reaction using the yeast tRNA<sup>Phe</sup> isolated from the cell was derived from the presence of m<sup>1</sup>A58 hypomodified tRNA<sup>Phe</sup>, as validated using a different primer extension method (Fig. 1B). Detection of a small amount of m<sup>1</sup>A58 hypomodified tRNA<sup>Phe</sup> is also consistent with m<sup>1</sup>A58 being nonessential for elongator tRNAs in yeast (Anderson et al. 1998). This result indicates that reverse transcription is a useful strategy for comparative analysis of m<sup>1</sup>A58 modification in tRNAs.

We applied a multistep strategy to study m<sup>1</sup>A58 hypomodification at the genomic scale (Fig. 1C). First, total RNA was isolated from human cell lines using standard methods. Two bacterial tRNA standards (*E. coli* tRNA<sup>Lys</sup>, *E. coli* tRNA<sup>Tyr</sup>) were then added to the total RNA. These bacterial tRNAs do not contain m<sup>1</sup>A58 (Sprinzl and Vassilenko 2005), and hence serve as internal loading and array hybridization controls between microarray measurements. Second, a mixture of RT primers were added that are complementary to nucleotides 76–59 for 40 families of human nuclear-encoded tRNAs (Dittmar et al. 2006) and three human mitochondrial-encoded tRNAs that are known to contain m<sup>1</sup>A58 modifications (Florentz et al. 2003), making 45 primers in all, including those for the *E. coli* tRNA standards. Primer extension was carried out using a low amount of Avian Myeloblastosis Virus (AMV) RT. The RT reaction was performed with [ $\alpha$ -<sup>32</sup>P]TTP to enable radioactive labeling of the RT products. After the RT reaction, the reaction mixture was treated with RNase H to remove the tRNA templates. The mixture was then filtered through size-exclusion columns to remove unincorporated [ $\alpha$ -<sup>32</sup>P]TTP in order to reduce the background of array hybridization. Following phenol-chloroform extraction and ethanol precipitation, the RT product mixture was hybridized directly on a custom-printed tRNA microarray (Netzer et al. 2009). The hybridized array was exposed to a PhosphorImager in order to visualize the RT products.

The microarray was designed with DNA probes having the same sequence as tRNA, hence complementary to the RT products. A total of 96 probes repeated eight times each were printed on the array. We applied stringent hybridization conditions to ensure that only long transcripts resulting from primer extension would hybridize on the array. In

general, these products range from RT stops at nucleotide 37 (~40-nucleotides [nt] long) where many human tRNAs contain elaborate modifications to RT run-off products (75–90-nt long) (Fig. 1D). Excess primers themselves (~18 mers) are not detected because the  $^{32}\text{P}$ -labeling requires primer extension. Using this design, numerous RT products were detected in a total RNA isolated from cultured human cells (Fig. 1E). In total, 136 spots showed signals above background, corresponding to 17 RT products.

We performed three control experiments to ensure that the majority of the signals on the arrays are derived from  $\text{m}^1\text{A58}$  hypomodified human tRNAs. First, we carried out the same experiment using just the primer sets plus the *E. coli* tRNA standards (Fig. 1E). In this case, only 40 spots corresponding to five RT products showed signals above background. As expected, the most intense signals (16 spots corresponding to two RT products) are derived from the two *E. coli* tRNAs. A total of 24 spots corresponding to three RT products can be attributed to products derived from the primers themselves; these products are not considered further in our subsequent analysis. Second, we tested the specificity of array hybridization by repeating the hybridization of the RT product in the presence of a molar excess of the Ala-hGC probe. The presence of this probe should compete only with the RT product derived from the Ala-hGC tRNAs. Indeed, signal from Ala-hGC tRNA is selectively diminished in the presence of this probe (Fig. 1F). Third, we carried out RT reaction with one primer at a time and analyzed the reaction product on denaturing PAGE (Fig. 1D). The reaction products detected in this way correspond nicely with those detected on the array. For example, high signal from Ala-hGC tRNA is detected by both array and gel methods. In contrast, signal from initiator-tRNA (Met-i) was at or below background in both array and gel experiments.

### $\text{m}^1\text{A58}$ hypomodification patterns in human cell lines

In order to obtain comprehensive information on  $\text{m}^1\text{A58}$  hypomodification patterns, we analyzed the total RNA extracted from five cell lines (Fig. 2). These cell lines represent a wide range of cell types: HeLa (cervical cancer), HEK293T (embryonic kidney), MDA-MB-231 (breast cancer, metastasized to lungs), MCF10A (human breast epithelium), and Neuroblastoma (adrenal gland).

tRNA from HeLa cells gave detectable RT signals for nine among 40 nuclear-encoded tRNA families and one among three mitochondrial-encoded tRNAs (Fig. 2A). Among the high signals were those obtained from tRNA<sup>Ala</sup>(hGC, h = I/C/U) and tRNA<sup>Pro</sup>(hGG). Both probes encompass several isoacceptors among Ala and Pro-tRNA families (IGC/CGC/UGC for Ala, and IGG/CGG/UGG for Pro). These isoacceptors have very similar sequences and cannot be separately detected in our array method (Dittmar et al. 2006). Three tRNAs with detectable signals are tRNA<sup>Asp</sup> and two isoacceptor families of tRNA<sup>Glu</sup>; these tRNAs have been

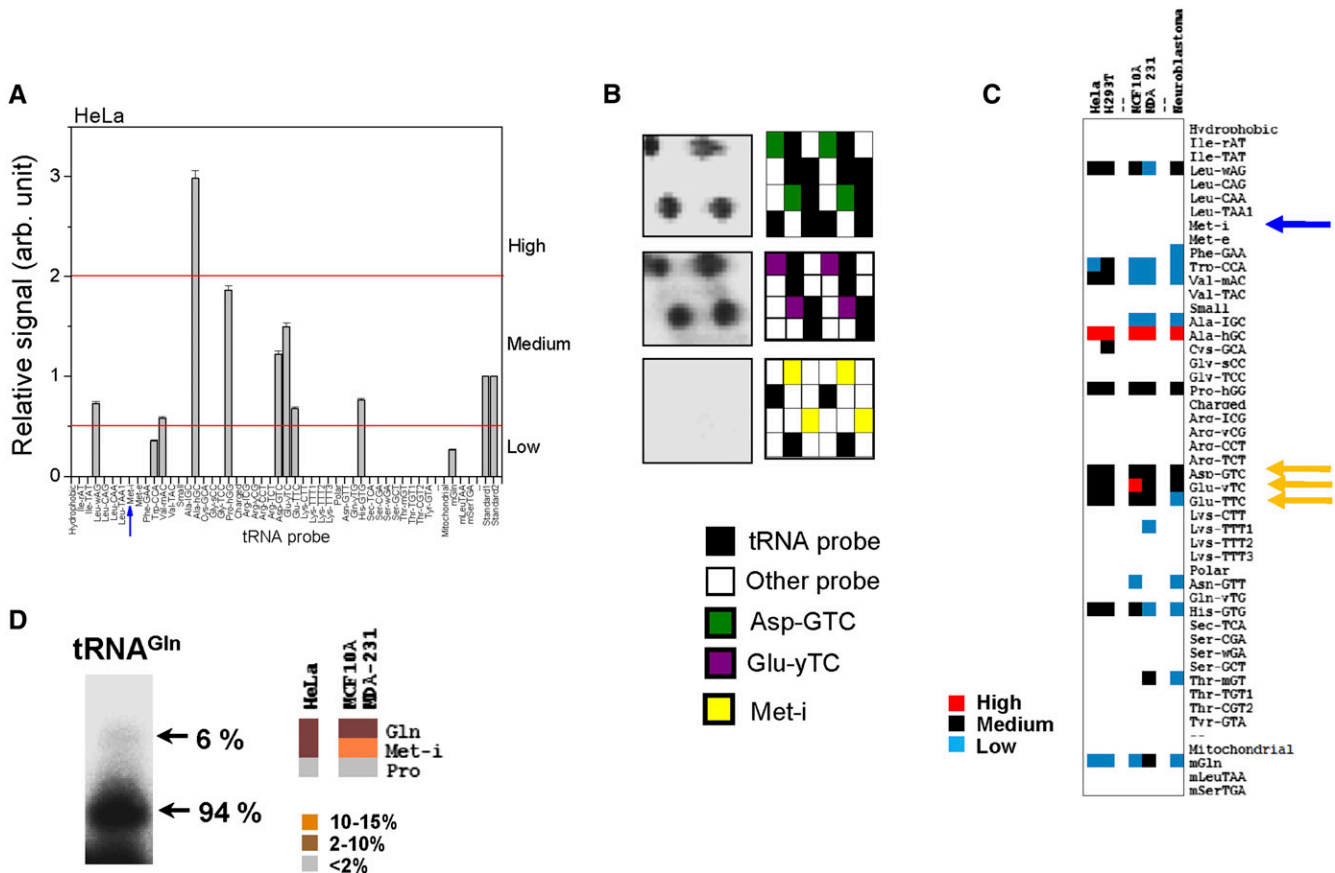
shown previously to be undermodified at A58 in rat cells (Kuchino et al. 1981; Chan et al. 1982), and therefore likely to be hypomodified in human cells as well. On the other hand, no signal was detected for the initiator-tRNA<sup>Met</sup> (Fig. 2A,B). In yeast,  $\text{m}^1\text{A58}$  is essential for the stability of the initiator-tRNA<sup>Met</sup>, and this requirement seems to extend to human initiator-tRNA as well. These results further validate our microarray approach in the detection of specific  $\text{m}^1\text{A58}$  hypomodified tRNAs.

It is much more difficult to determine the absolute fraction of  $\text{m}^1\text{A58}$  hypomodification for each tRNA in the array experiment. Major variables include the differences in the efficiency of the RT reaction and array hybridization for each tRNA. For normalization purposes, we denoted  $\gamma = 1.0$  as the array signals detected from the two *E. coli* tRNA standards. This value corresponds to 0.066 pmol of each *E. coli* tRNA standard added to 1  $\mu\text{g}$  of total human RNA (Fig. 2A). By this measure, we group the hypomodified tRNA species in three groups: high ( $\gamma > 2$ ), medium ( $\gamma = 0.5\text{--}2$ ), and low ( $\gamma < 0.5$ ). For HeLa cells, the high group includes one member (Ala-hGC), the medium group has seven members (Pro-hGG, Asp-GTC, Glu- $\gamma\text{TC}$ , Glu-TTC, Leu-wAG, Val-mAC, His-GTG), and the low group has two members (Trp-CCA, mitochondrial-Gln).

The pattern of the  $\text{m}^1\text{A58}$  hypomodified tRNAs is similar in five human cell lines (Fig. 2C). The same set of 10 tRNAs hypomodified in HeLa cells is hypomodified in all other cell lines as well, and the extent of hypomodification varies little. In all cases, no detectable signal was obtained for the initiator-tRNA (Fig. 2C, indicated by the blue arrow), and detectable signals were always obtained for Asp and Glu-tRNAs (Fig. 2C, indicated by the orange arrows). These results suggest that human cells have a basal pattern of  $\text{m}^1\text{A58}$  hypomodification, and this basal pattern includes ~25% of human tRNA species.

In addition to these 10 common tRNAs, other cell lines appear to have one (HEK293, MCF10A), two (MDA-MB-231), and four (Neuroblastoma) more tRNAs that are hypomodified. Hypomodification signals from these non-common tRNAs generally belong to the low group. They may play some unique roles that are specific for each cell type.

The MCF10A and MDA-MB-231 cell lines are often used for comparative studies between nontumorigenic versus tumorigenic breast cells (Chen et al. 1997; Gruber and Pauli 1999; Mehta et al. 2004). The nuclear-encoded tRNAs are overexpressed by approximately twofold, on average, in MDA-MB-231 over MCF10A cells (Pavon-Eternod et al. 2009). These cell lines have a similar pattern and extent for  $\text{m}^1\text{A58}$  hypomodification, therefore suggesting that the extent of hypomodification is maintained in breast cancer cells. Interestingly, MDA-MB-231 appears to contain 1.7–1.8 times more  $\text{m}^1\text{A58}$  methyltransferase subunits, TRM6 and TRM61, compared with MCF10A (Fig. 3; data not shown). This result suggests that tRNA overexpression is also accompanied by an



**FIGURE 2.** Profiling m<sup>1</sup>A58 hypomodification in human cell lines. (A) Hypomodified tRNA species in HeLa cells; the blue arrow indicates initiator-tRNA<sup>Met</sup>, which is completely modified at A58. The relative signal ( $y$ -axis) = 1 are those of the two tRNA transcripts that are unmodified at A58. For this work, we defined that  $y < 0.5$  has low,  $y = 0.5-2$  has medium, and  $y > 2$  has high levels of hypomodified tRNA. (B) Array image from cell line HeLa showing the signals for Asp-GTC and Glu-yTC, and the absence of signal for Met-i. (C) Heat map showing ranges (high, medium, low) of m<sup>1</sup>A58 hypomodification in five human cell lines (HeLa, HEK 293T, MDA-MB-231, MCF-10A, and Neuroblastoma). The blue arrow indicates initiator-tRNA<sup>Met</sup> (negative control) and the orange arrows indicate tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> (positive controls). (D) Determination of hypomodification at m<sup>1</sup>G9 for tRNA<sup>Gln</sup>, tRNA<sup>Met</sup> and tRNA<sup>Pro</sup>. The detection limit for hypomodification is ~2% in this experiment. The gel shows the primer extension reaction for tRNA<sup>Gln</sup> in the total RNA from MDA-MB-231.

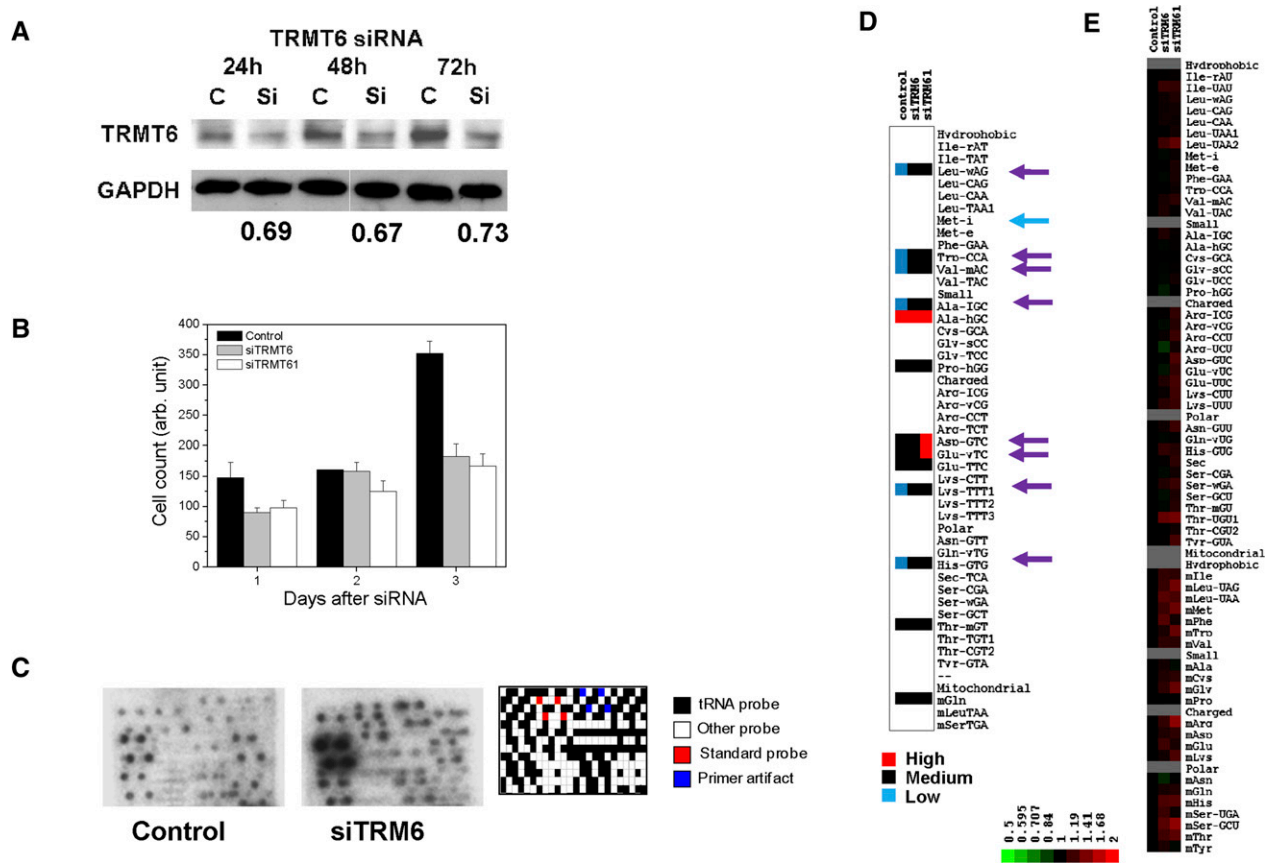
increase in methyltransferase levels, presumably to ensure that no significant amount of m<sup>1</sup>A58 hypomodified tRNA accumulates in the cell.

In order to determine whether human tRNAs are also hypomodified at other positions, we performed primer extension to examine the known 1-methyl-G modification at position 9 for three tRNAs (Bjork et al. 2001; Jackman et al. 2003) in three cell lines (Fig. 2D). For m<sup>1</sup>G9 modification, the primers are complementary to nucleotides 33–14 of these tRNAs. Using three dNTPs and one ddNTP, primer extension could generate two products, the shorter product corresponding to the reverse transcriptase stop at the m<sup>1</sup>G9 modification, and the longer product corresponding to the incorporation of the dideoxy-nucleotide (Sanger et al. 1977). The longer product is only observed when the tRNA is hypomodified at m<sup>1</sup>G9. Like m<sup>1</sup>A58, we found that m<sup>1</sup>G9 is also hypomodified at varying amounts for each tRNA, and each tRNA has a similar hypomodification pattern among the three cell lines. This result suggests that human cells con-

tain distinct patterns of tRNA hypomodification at distinct modification sites.

### siRNA knockdown of m<sup>1</sup>A58 methyltransferase subunits

To examine the consequence of m<sup>1</sup>A58 hypomodification and further establish our array method as a suitable tool for functional studies, we performed siRNA experiments on either of the human m<sup>1</sup>A58 methyltransferase subunits, TRM6 or TRM61 (Ozanick et al. 2005). We chose to carry out this experiment with MDA-MB-231 cells because tRNAs are known to be overexpressed in these cells (Pavon-Eternod et al. 2009), and this high amount of tRNA may be more sensitive to variations in this modification enzyme. siRNA treatment reduced the mRNA levels of both methyltransferase subunit genes by ~10-fold compared with the siRNA control (data not shown); however, the reduction in protein level is less than twofold (Fig. 3A). This difference between



**FIGURE 3.** Effect of siRNA knockdown of m<sup>1</sup>A-methylase subunits, TRM6 and TRM61. (A) Western blot showing efficiency of siRNA treatment of MDA-MB231 cells. The siRNA treatment causes the decrease in protein level after 3 d of treatment. GAPDH level is used as a control. (B) Growth of control versus TRM6 and TRM61 siRNA-treated cells. (C) Representative array image from control and siTRM6-treated cells. (D) Heat map of hypomodification in control versus TRM6 and TRM61 knockdown cells. Purple arrows show tRNA species with significant increase in hypomodification in the siRNA-treated cells. Blue arrow indicates initiator-tRNA<sup>Met</sup>, which, as expected, is not hypomodified at all times. (E) Heat map of relative tRNA abundance of the control and siRNA-treated cells shows no change in tRNA abundance in all cases.

changes in mRNA and protein levels of the m<sup>1</sup>A58 methyltransferase may be either derived from an unknown translational regulation for this multisubunit enzyme, or this enzyme may turn over very slowly. Nevertheless, this moderate reduction of the methyltransferase levels led to slower cell growth as compared with siRNA-control cells after 3 d (Fig. 3B), suggesting that this enzyme plays a role in cell proliferation. Observing the growth phenotype only after 3 d may be related to the longevity of tRNAs in mammalian cells, so that a time scale of days is needed to produce a significant change in the tRNA population.

We then determined the changes in the m<sup>1</sup>A58 modification levels in cells treated with siRNA targeted for either subunit of this methyltransferase (Fig. 3C,D). We found significantly higher levels of hypomodification for eight tRNAs that are already hypomodified in control cells. Again, initiator-tRNA<sup>Met</sup> remains fully modified in all cases. Furthermore, very little new hypomodified tRNA species are present significantly above background in the siRNA-treated cells. On the other hand, the amount of all tRNA species as measured by our standard tRNA microarray method using

Cy3 and Cy5 labeled total tRNA samples (Dittmar et al. 2006; Pavon-Eternod et al. 2009, 2010; Zhou et al. 2009) in the siRNA-treated methyltransferase cells remains essentially unchanged as compared with siRNA-control cells after 3 d (Fig. 3E). A plausible explanation of the unchanging level of these tRNAs is that since the reduction of the methyltransferase level is less than twofold, a sufficient amount of the methyltransferase enzyme remains after siRNA treatment to still enable full modification of these tRNAs. Compared with the m<sup>1</sup>A58 hypomodified tRNAs, the fully modified tRNAs may be more efficient substrates for the methyltransferase enzyme.

There are several plausible explanations for the apparent slow growth phenotype. The phenotype may be derived from an overproduction of hypomodified tRNA species that is toxic to the cell. The phenotype may also be due to impairment of additional functions of the methyltransferase enzyme in the cell. Furthermore, the methyltransferase may act on yet-to-be-identified substrates that are not modified and are required for efficient cellular replication. Regardless, these results suggest that the presence of m<sup>1</sup>A58

hypomodified tRNAs in untreated cells are due to their inherent stability and not due to insufficient cellular tRNA degradation capacity.

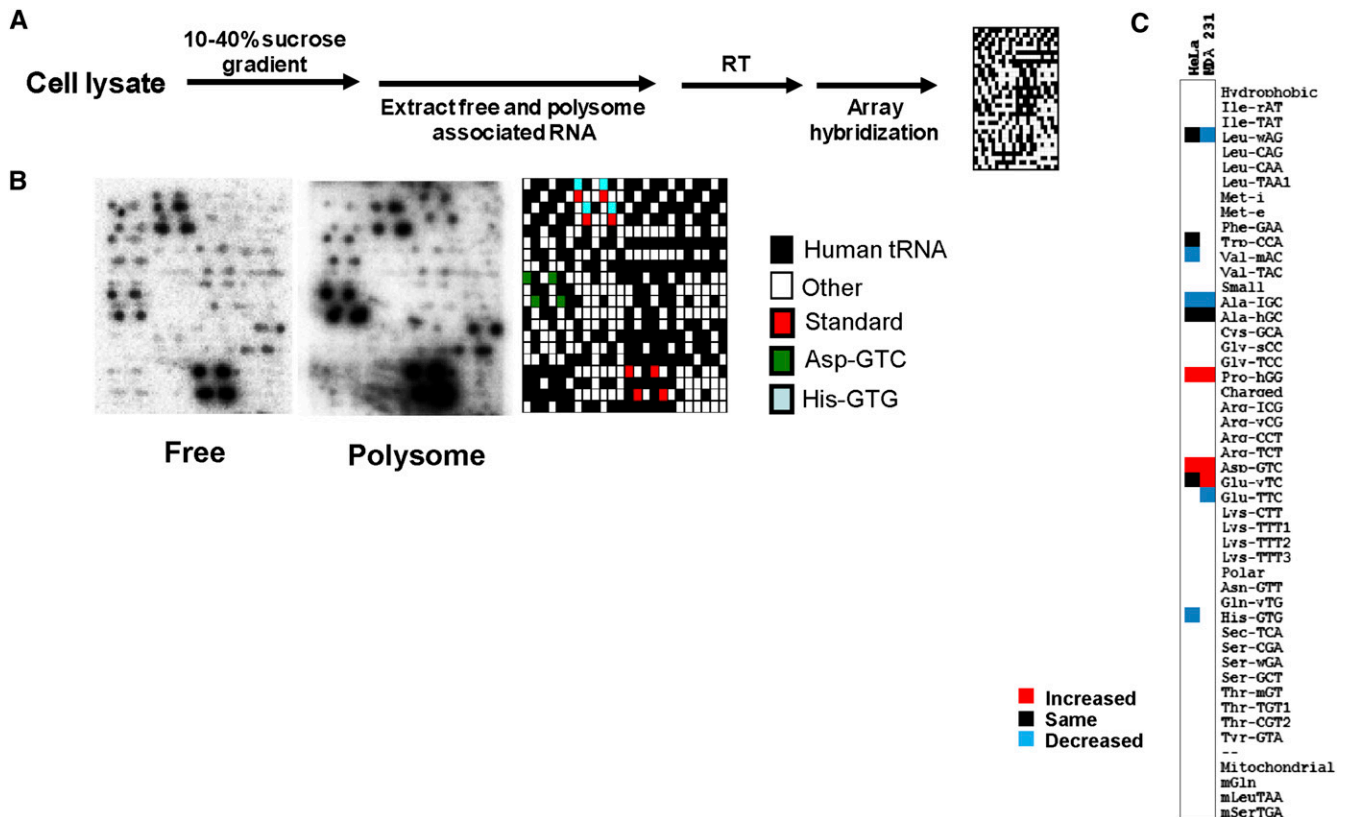
### Polysome association of m<sup>1</sup>A58 hypomodified tRNAs

Participation in translation for a tRNA requires its association with the polyribosomes. m<sup>1</sup>A58 modification could have a strong effect for polysome association because this region of tRNA interacts with the ribosome during translation (Ramakrishnan 2002; Korostelev and Noller 2007). In order to determine how m<sup>1</sup>A58 hypomodified tRNAs associate with polysomes, we extracted total RNA from free (low-sucrose density) and polysome-bound (high-sucrose density) fractions in a sucrose density gradient and performed RT reaction with these RNAs after the addition of *E. coli* tRNA standards (Fig. 4A).

In HeLa cells, signals for eight of nine nuclear-encoded tRNAs are present in both free and polysome fractions (Fig. 4B,C). The mitochondrial tRNA<sup>Gln</sup> was not detected in this experiment. We analyzed the array data by first dividing the amount of the RT product from polysome fractions by the free-tRNA fraction for each tRNA. This ratio was then nor-

malized to the signals from the two *E. coli* tRNA standards. Among the nine tRNAs, the median value generated this way is 0.83 (mean value = 0.90 ± 0.59) which is close to the value of 1.0. Only four of the nine tRNAs show ratios within 1.5-fold of the median value generated in this way. Assuming the same experimental variation of 1.5-fold when comparing multiple microarray data, this result suggests that m<sup>1</sup>A58 hypomodification has a significant effect on ribosome association of these tRNAs. Two of the nine tRNAs, Pro-hGG and Asp-GTC, show ratios approximately twofold above median. For these two tRNAs, their m<sup>1</sup>A58 hypomodified species seem to be enriched in polysome. In contrast, three of the nine tRNAs, Val-mAC, Ala-IGC, and His-GTG, show ratios more than twofold below median. The hypomodified version of these three tRNAs seems to be depleted in polysomes.

In MDA-MB-231 cells, signals for seven out of 12 nuclear-encoded tRNAs are present in both free and polysome fractions (Fig. 4C). Polysome enrichments or depletion for these detectable hypomodified tRNAs are similar between these two cells lines. In particular, Pro-hGG and Asp-GTC also show high ratios in polysome-associated fractions in MDA-MB-231 cells, validating the similar result observed in HeLa cells.



**FIGURE 4.** Effect of m<sup>1</sup>A hypomodification on polysome association. The analysis was performed with HeLa and MDA-MB231 cells. (A) Scheme of the procedure to measure polysome association. (B) Array images showing differences between free and polysome samples from HeLa cells. The array key is on the right. (C) Heat map of polysome association. Color coding shows the comparison of polysome association of m<sup>1</sup>A58 hypomodified tRNA with fully modified tRNA.

## DISCUSSION

Eukaryotic tRNAs contain a large number of post-transcriptional modifications. Most modifications are not essential for cell viability, and each modification can incrementally affect tRNA function at multiple levels, including maturation, aminoacylation, and ribosome utilization (Bjork 1995). Despite many years of studies, an open question remains on the identity and frequency of hypomodified tRNAs that are present in the cell. Using a functional genomics approach, we demonstrate in this work that ~25% of human tRNA species are hypomodified at the conserved A58 nucleotide. The identity of m<sup>1</sup>A58 hypomodified tRNA species is similar in five human cell lines examined, suggesting that human cells share a common pattern of m<sup>1</sup>A58 hypomodification. As positive controls for our genomic approach, we identified that the initiator-tRNA<sup>Met</sup> is fully modified, and the tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> species are hypomodified in all five cell lines, in agreement with the literature results (Kuchino et al. 1981; Chan et al. 1982). It is an open question as to whether m<sup>1</sup>A58 modification is essential for the maturation of the majority of human tRNAs or whether fully modified tRNAs are simply more efficient substrates for the m<sup>1</sup>A58 methyltransferase.

The human tRNA species that are m<sup>1</sup>A58 hypomodified are distributed among distinct tRNA families. At this time, no obvious pattern derived from common sequence or alternate secondary structure motifs can be deduced for these tRNAs (data not shown). Nevertheless, the presence of m<sup>1</sup>A58 hypomodified tRNAs in human cells clearly indicate that this modification is not required for the maturation of all tRNAs. Three of the 22 mitochondrial-encoded tRNAs also undergo m<sup>1</sup>A58 modifications (Sprinzl and Vassilenko 2005), although, to our knowledge, the m<sup>1</sup>A58 methyltransferase in human mitochondria has not been identified. Here, we tested whether m<sup>1</sup>A58 hypomodification also occurs in these three mitochondrial tRNAs, two of which (mLeu-UAA; mGln) possess all features needed for a classical cloverleaf secondary structure (Helm et al. 1998; Florentz et al. 2003; Sprinzl and Vassilenko 2005). Only mitochondrial tRNA<sup>Gln</sup> among these three tRNAs is hypomodified, and this hypomodification pattern is again conserved among the seven cell lines tested. The functional significance of the m<sup>1</sup>A58 hypomodification in mito-tRNA<sup>Gln</sup> is unclear.

Although m<sup>1</sup>A58 hypomodified tRNAs are inherently stable in human cells, in order to participate in translation, these tRNAs have to be aminoacylated. Because A58 is conserved in all tRNAs, this residue is not used directly as an identity element for aminoacyl-tRNA synthetases (Giege et al. 1998). Some synthetases, however, do use residues 59 and 60 as a part of their identity elements; for example, the *E. coli* aminoacyl-tRNA<sup>Phe</sup> synthetase. m<sup>1</sup>A58 modification with an extra positive charge and the presence of an extra methyl group may influence the charging efficiency of these synthetases.

We do not know at this time why human cells maintain a subset of tRNAs that are m<sup>1</sup>A58 hypomodified. However, several hypotheses can be proposed for their utilization.

1. Neutral/Null hypothesis: The hypomodified tRNAs are less-efficient substrates for the m<sup>1</sup>A58 methyltransferase enzyme compared with the other, fully modified tRNAs. Since most of the hypomodified tRNAs are still functional in polysome association, their presence is simply tolerated. This hypothesis would be consistent with the observed growth phenotype being derived from an unknown m<sup>1</sup>A58 methyltransferase function not related to tRNA methylation.
2. Nontranslation hypothesis: The hypomodified tRNAs are used for a function not involving mRNA decoding. Because N<sup>1</sup>-methylation at A58 introduces a positive charge in the elbow region of tRNA, the absence of this modification in some tRNAs may decrease its binding by the elongation factor eEF1 $\alpha$  or by the ribosome. This decreased binding efficiency reduces competition by the major cellular components of translation, allowing the hypomodified tRNAs to be used as ligands for other proteins, e.g., the protein kinase GCN2 (Hinnebusch 2005).

Another possibility for keeping hypomodified tRNAs in the cell is to provide a reservoir for tRNAs that may be readily altered in stress response. For instance, many types of stresses in yeast and in mammalian cells generate tRNA fragments derived from cleavage of the anticodon loop (Thompson and Parker 2009a,b; Yamasaki et al. 2009). The amount of these tRNA fragments is small and does not affect the abundance of these tRNAs, yet these fragments alone can lead to translational repression in a mammalian cell (Yamasaki et al. 2009).

3. Speed-accuracy modulation hypothesis: Fully modified tRNAs often increase translation accuracy (Bjork 1995), but the increase in accuracy can reduce the overall speed of translation. The utilization of some hypomodified tRNAs may be useful to reduce the “bottleneck” in translation, allowing faster translation speed, and to balance that with sufficient accuracy (Drummond and Wilke 2008).

This brief discussion implies that the function of m<sup>1</sup>A58 modification may not be singular. It is plausible that m<sup>1</sup>A58 modification, even though occurring at the same position in all tRNAs, can confer different functions depending on the tRNA species. Additional possibilities for m<sup>1</sup>A58 function include dynamic changes of its hypomodification pattern as a function of cell cycle, cell density, or cell states. Furthermore, hypomodification patterns may also change in response to various types of stresses that cells may encounter. The ability to measure the hypomodification pattern at the genomic level as described here should provide a valuable tool to further



examine dynamic variations and potential functions of this modification for individual tRNAs.

## MATERIALS AND METHODS

### RNA preparation

Total RNA from the HeLa, HEK293, MCF10A, MDA-MB-231, and Neuroblastoma cell lines were obtained using the mirVana miRNA Isolation Kit (Ambion, <http://www.ambion.com>) according to manufacturer's manual. The two tRNA standards, *E. coli* tRNA<sup>Lys</sup> (No. R6018) and *E. coli* tRNA<sup>Tyr</sup> (No. R0258) and the yeast tRNA<sup>Phe</sup> (No. R4018) were purchased from Sigma-Aldrich (<http://www.sigmaaldrich.com>) and used without further purification. The yeast tRNA<sup>Phe</sup> transcript was obtained by T7 RNA polymerase transcription. The DNA oligos for primer extension were synthesized by Integrated DNA Technologies (IDT, <http://www.idt.com>). All DNA primers were purified by denaturing PAGE and stored in water.

### Primer extension for array studies

The primer extension was carried out with 2 µg of total RNA, 1 pmol of each primer, 0.13 pmol each of *E. coli* tRNA<sup>Lys</sup> and tRNA<sup>Tyr</sup> in a total volume of 10 µL. The RNA and primers were annealed by heating at 90°C for 4 min with annealing buffer (50 mM Tris-HCl at pH 7.5 and 3 mM KCl) and the mixture was allowed to gradually cool to 40°C over a period of 30 min. The RT reaction was carried out at 43°C with 1 mM each dATP, dGTP, dCTP, 0.5 mM dTTP, and spiked with radioactive [<sup>32</sup>P]TTP and 2 U of AMV RT (USB, Inc.) in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.5 mM Spermidine, and 10 mM DTT for 20 min. The mixture was further treated with 0.1 U/µL RNase H (from Epicenter Technologies) at 43°C for 10 min, filtered through G-25 columns to remove excess [<sup>32</sup>P]TTP (GE Technologies), followed by phenol:chloroform extraction (to remove all enzymes) and ethanol precipitation. The pellet was dissolved in 10 µL of water, and 1 µL was loaded on a denaturing polyacrylamide gel containing 7 M urea to visualize RT products.

### Primer extension for yeast tRNA<sup>Phe</sup> and m<sup>1</sup>G9 modification studies

The primer extension was carried out in a total volume of 10 µL with 1 µg of total RNA and 1 pmol of designated 5' <sup>32</sup>P-labeled primer. For yeast tRNA<sup>Phe</sup>, the primer was complementary to nucleotides 76–60; for human tRNAs, the primers were complementary to nucleotides 33–14 of the corresponding tRNA. The RNA and primers were annealed by heating at 90°C for 4 min with annealing buffer (50 mM Tris-HCl at pH 7.5 and 3 mM KCl) and the mixture was allowed to gradually cool to 40°C over a period of 30 min. Each RT reaction was carried out at 43°C with 1 mM each of three dNTPs (dA/C/TTP for yeast tRNA<sup>Phe</sup> and human tRNA<sup>Gln</sup>; and dA/C/GTP for human initiator-tRNA<sup>Met</sup> and human tRNA<sup>Pro</sup>), 1 mM of one ddNTP (ddGTP for yeast tRNA<sup>Phe</sup> and human tRNA<sup>Gln</sup>; and ddTTP for human initiator-tRNA<sup>Met</sup> and human tRNA<sup>Pro</sup>), and 1 U of AMV RT (USB, Inc.) in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.5 mM Spermidine, and 10 mM DTT for 20 min. The mixture was treated with 0.1 U/µL RNase H (Epicenter Technologies) at 43°C for 10 min. The reaction was quenched with the addition of an equal volume of 9 M urea/50 mM EDTA, and the RT product separated on denaturing polyacrylamide gels.

### Polysome association measurements

To isolate RNA for polysome analysis, the cells with a confluence of 30%–50% were aspirated and washed twice with 1X PBS. Cells were scraped off the plates after adding lysis buffer containing a 2.5% (v/v) mixture of Nonidet P-40 in 0.2 M sucrose in 50 mM Tris (pH 7.5), 100 mM ammonium acetate, 5 mM MgCl<sub>2</sub>, and 3 mM β-mercaptoethanol was added to the plates. The cell lysate was centrifuged at 4°C for 30 min. The clear supernatant was collected and was loaded on top of a 10%–40% sucrose gradient. The gradient was prepared by dissolving ultra pure sucrose (Sigma Aldrich) in a gradient buffer consisting of the same buffer as above.

### Hybridization and microarray analysis

The tRNA microarray for m<sup>1</sup>A58 modification contained a total of 95 oligonucleotide probes plus one buffer only slot repeated eight times each. The array contains 59 sense probes for human chromosomal-encoded tRNAs, 22 sense probes for human mitochondrial-encoded tRNAs, plus eight antisense tRNA probes from hybridization controls and six nonhuman tRNA probes as normalization controls. The oligonucleotides were printed manually to generate large array spots necessary for radioactive detection (Netzer et al. 2009). Array hybridization has been described previously for the analysis tRNA abundance and charging levels (Dittmar et al. 2005, 2006). A brief description follows. Immediately prior to hybridization, microarray slides were immersed in boiling water for 2 min to remove undesired particles and uncross-linked oligos. The reverse-transcribed RNA samples (corresponding to ~2 µg of total RNA) were dissolved in microarray hybridization buffer (Sigma Aldrich) containing 20 µg of salmon sperm DNA and 10 µg of poly(A). The sample was applied to the Hybridization chamber of the GeneTAC Hyb4 station (Genomic solutions). The following program was used for hybridization: 75°C (2 min), 60°C (probe introduction), 90°C (5 min), 60°C (16 h). The slides were then washed on the Hyb4 station twice with 2XSSC, 0.1% (w/v) SDS at 50°C, twice with 0.1X SSC, 0.1% (w/v) SDS at 42°C, and twice with 0.1X SSC at 42°C. Slides were then removed from the station, rinsed with 0.1XSSC, and dried by centrifugation.

Microarray slides were imaged using a FUJI BAS scanner. <sup>32</sup>P-intensities were quantified and background-subtracted using Fuji BAS software. Median values for eight replicate spots were used for further analysis.

### Cell culture, siRNA transfection, and cell growth

MDA-MB-231 cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 (Mediatech) with 10% FBS (GIBCO), 100 U/mL Penicillin, and 100 µg/mL Streptomycin. Transfection of control siRNA (Dharmacon), TRM61, and TRMT6 siRNA (Santa Cruz Biotechnology) by Lipofectamine RNAiMAX (Invitrogen) was performed according to the manufacturer's protocol in 96-, 24-, and 6-well format. The cells were plated in RPMI 1640 with 10% FBS for 24 h. The cells with a confluence of 30%–50% were washed twice and changed to Opti-MEM (GIBCO) without serum before transfection was performed. For a 24-well format transfection, 12 nmol siRNA (final concentration of 20 nM) and 1.25 µL of RNAiMAX were used. The medium was changed back to RPMI 1640/10% FBS after 24 h. Cell growth curve was measured by CellTiter-Blue Cell Viability Assay (Promega) with an incubation time of 3 h at the time point of measurement.

## qRT-PCR and Western blot analysis

Total RNA was isolated with the mirVana miRNA Isolation Kit (Ambion). qRT-PCR was carried out using the SuperScript III Platinum One-Step qRT-PCR System (Invitrogen) according to the manufacturer's protocol in the Applied Biosystems AB7300 system.

Western blot analysis was performed in a 4%–12% Tris-HCl gel, with a 1:400 dilution of TRMT6 (H-198), and TRM61 (C145I165) antibody (Santa Cruz Biotechnology) with the corresponding 1:1000 dilution of HRP conjugated secondary antibody (GE Healthcare).

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