Subcellular relocalization and nuclear redistribution of the RNA methyltransferases TRMT1 and TRMT1L upon neuronal activation

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ABSTRACT
RNA modifications are dynamic chemical entities that expand the RNA lexicon and regulate RNA fate. The most abundant modification present in mRNAs, N6-methyladenosine (m\textsuperscript{6}A), has been implicated in neurogenesis and memory formation. However, whether additional RNA modifications may be playing a role in neuronal functions and in response to environmental queues is largely unknown. Here we characterize the biochemical function and cellular dynamics of two human RNA methyltransferases previously associated with neurological dysfunction, TRMT1 and its homolog, TRMT1-like (TRMT1L). Using a combination of next-generation sequencing, LC-MS/MS, patient-derived cell lines and knockout mouse models, we confirm the previous reported dimethylguanosine (m\textsuperscript{2,2}G) activity of TRMT1 in tRNAs, as well as reveal that TRMT1L, whose activity was unknown, is responsible for methylating a subset of cytosolic trNA\textsuperscript{AAU}(AGC) isocodons at position 26. Using a cellular in vitro model that mimics neuronal activation and long term potentiation, we find that both TRMT1 and TRMT1L change their subcellular localization upon neuronal activation. Specifically, we observe a major subcellular relocalization from mitochondria and other cytoplasmic domains (TRMT1) and nucleoli (TRMT1L) to different small punctate compartments in the nucleus, which are as yet uncharacterized. This phenomenon does not occur upon heat shock, suggesting that the relocalization of TRMT1 and TRMT1L is not a general reaction to stress, but rather a specific response to neuronal activation. Our results suggest that subcellular relocalization of RNA modification enzymes may play a role in neuronal plasticity and transmission of information, presumably by addressing new targets.

Introduction
Epigenetic processes involving DNA methylation and histone modifications are vital for learning and memory formation [1]. RNA modifications, which are much less characterized, add an additional and largely unexplored layer of regulation and avenue for environmental response in cellular function [2–9]. Dysregulation of the RNA modification machinery has already been linked to many human diseases [10–13], including several neurological diseases [14–20]. A number of studies have pointed to RNA modifications as potential molecular mechanisms underlying the experience-dependent plasticity of the brain [2,7,21–23], but a systematic characterization of which RNA modifications may be playing a role in neuronal plasticity and response to environmental signals is lacking.

Cognitive impairment of intellectual disability (ID) is a genetically heterogeneous neurodevelopmental disorder, estimated to affect 2–3% of the population [24]. Mutations in three RNA methyltransferase-encoding genes have been linked to intellectual disability, suggesting an important role for RNA modifications in the development of cognitive functions. These include the 2′-O-methyltransferase FTSJ1, identified in X-linked non-syndromic intellectual disability in which mental retardation is the sole clinical feature [25,26], as well as the m\textsuperscript{6}C methyltransferase NSUN2 and the m\textsuperscript{2,2}G methyltransferase TRMT1, both of which have
been identified as the cause of autosomal-recessive intellectual disability (ARID) [27–30].

TRMT1 is an RNA methyltransferase responsible for the formation of N2,N2-dimethylguanosine (m^2,2-G) at position 26 in both cytosolic and mitochondrial tRNAs [31,32]. In vertebrates, TRMT1 has a homolog, TRMT1-like (TRMT1L), in which the methyltransferase domain is conserved [33]. The enzymatic activity of human TRMT1 has been previously demonstrated [31,32], but the function and targets of TRMT1L are unknown. Similarly to TRMT1, the TRMT1L gene is involved in cognitive functioning [33]. Indeed, knock-out of TRMT1L in mice has been shown to cause altered motor coordination and aberrant exploratory behaviour [33], suggesting that its activity is also associated with neurological functions.

To dissect the biological role of TRMT1L, here we employed a combined strategy of Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and next-generation sequencing (NGS) to map m^2,2-G modifications both in long and short RNAs in TRMT1L knock-out and wild type mouse brain samples. As a control, we also analysed the m^2,2-G modifications in TRMT1-deficient and control cell lines. Through this analysis, we confirm the previously reported dimethylase activity of TRMT1 in cytosolic and mitochondrial tRNAs [31,32], as well as show that TRMT1L specifically dimethylates a subset of tRNA^Ala (AGC) isodecoders at position 26.

Using a cellular in vitro model that mimics neuronal activation and long term potentiation [34], we examine the behaviour of these RNA methyltransferases, finding that both TRMT1 and TRMT1L change their subcellular localization upon neuronal activation. Specifically, we observe a major subcellular relocalization from mitochondria and other cytoplasmic domains (TRMT1) and nucleoli (TRMT1L) to different small punctate compartments in the nucleus, which are as yet uncharacterized. This phenomenon does not occur upon stresses such as heat shock, suggesting that the relocalization of TRMT1 and TRMT1L is not a general reaction to stress, but rather a specific response to neuronal activation.

Results

Evolutionary analysis of the human m^2,2-G modification machinery: TRMT1 and TRMT1L

Human TRMT1 and TRMT1L only share 20% similarity in their DNA sequence [32]; however, their domain structure is relatively similar, including a conserved homologous methyltransferase domain (Fig. 1A). Whilst the dimethylguanosine transferase activity of TRMT1 has been previously reported [31,32], the activity of TRMT1L is unknown.

To identify the full set of enzymes potentially capable of placing m^2,2-G in the human transcriptome, we performed a Hidden Markov Model (HMM)-based search of the m^2,2-G methyltransferase domain (Pfam domain annotation: TRM), finding that only TRMT1 and TRMT1L showed statistically significant matches in human (see Methods). We

Figure 1. Characterization of the m^2,2-G modification machinery. (A) Chemical structure and dimethylation reaction catalysed by TRMT1 and potentially by TRMT1L is depicted. On the right part of the panel, a schematic representation of the domains found in proteins containing TRM1 methyltransferase domain (TRMT1L, TRMT1 and TRM1) is shown. The canonical isomorf has been chosen to depict each protein. See also Figure S2 for predicted domains in individual isomorphs. (B) Phylogenetic tree of eukaryotic proteins with homology to the TRM1 methyltransferase domain, showing that TRMT1L proteins are paralogs of TRMT1. (C) Immunoblotting of TRMT1L and TRMT1 in human SH-SYSY cells, which were fractionated into nuclear and cytoplasmic content. H3 was used as a nuclear marker, whereas GAPDH was used as cytoplasmic marker. Biological triplicates are shown. See also Figure S3 for uncropped immunoblot images. (D,E) Immunofluorescence of TRMT1 (D) and TRMT1L (E) in SH-SYSY cells. Nucleus has been marked with DAPI, cytoplasm has been labelled using anti-phalloidin antibody, mitotracker has been used to label the mitochondria, and anti-fibrillarin has been used to label the nucleolus. See also Figure S4 for immunofluorescence results in HeLa and HEK293.
found that the duplication of TRMT1 occurred at the base of vertebrates, originating TRMT1L (Fig. 1B), which was retained in all vertebrate species analysed. From our analyses, we find that the TRM m^{2,2}G methyltransferase domain is found in all eukaryal and archael species analysed, as previously reported [35], but also in a limited set of bacterial phyla (Aquificales and Cyanobacteria), likely acquired via horizontal gene transfer (Figure S1).

**TRMT1 and TRMT1L have different subcellular localizations**

TRMT1 and TRMT1L are predicted to reside in different organelles based on their signal peptides (Fig. 1A, see also Figure S2). Specifically, TRMT1 is predicted to contain a mitochondrion-targeting peptide signal, and thus is likely driving m^{2,2}G methylation of mitochondrial RNAs, whereas a second TRMT1 isoform is predicted to remain in the cytoplasm (Figure S2, see also Methods). By contrast, TRMT1L contains a predicted strong NLS signal, and is therefore expected to reside in the nucleus.

To confirm the subcellular localization predictions, both Western Blot and immunofluorescence assays were performed. We observed that TRMT1 was located in punctate domains in the cytoplasm and in the mitochondria of neuroblastoma-derived SH-SY5Y cells (Fig 1C,D see also Figure S3), whereas HeLa cells showed also nuclear localization in addition to cytoplasmic and mitochondrial localization (Figure S4A). The observed localizations are in agreement with the previously described activity of TRMT1 on both cytosolic and mitochondrial tRNAs [31,32]. By contrast, TRMT1L in SH-SY5Y cells was mainly localized in the nucleus (Fig 1E), in agreement with previous works studying TRMT1L localization in HeLa [31], and more specifically, colocalizing with nucleoli (Fig. 1E). To further validate the subcellular localization of TRMT1 and TRMT1L as well as the specificity of the respective TRMT1 and TRMT1L antibodies used, we performed a transfection for transient expression of TRMT1-FLAG and TRMT1L-FLAG in HEK293 cells (see Methods), finding that TRMT1 and TRMT1L stainings colocalized with those obtained using anti-FLAG in transfected cells (Figure S4B,C). Altogether, the distinct subcellular locations of these two enzymes suggest that TRMT1 and TRMT1L are addressing different RNA targets.

**M^{2,2}G is found in tRNAs, but also in higher RNA fractions in neuronal cell lines and tissues**

Early in vitro studies in human cells showed that TRMT1 is responsible for placing m^{2,2}G in numerous tRNAs at position 26 [32]. More recently, using CRISPR gene knockout systems, it was shown that TRMT1 deletion leads to a loss of m^{2,2}G in cytoplasmic and mitochondrial tRNAs [31], confirming its activity in a cellular context. However, whether TRMT1 places m^{2,2}G in substrates other than tRNAs, and what the activity and biological targets of TRMT1L may be, are still open questions.

To decipher whether m^{2,2}G is found beyond tRNAs, we performed LC-MS/MS analysis of specific RNA pools. Total RNA samples were fractionated into 5 different pools (see Methods), corresponding to: (i) total RNA fraction (containing mostly tRNAs), (ii) ribodepleted polyA(+) fraction (containing mostly mRNAs), (iii) ribodepleted non-polyA(+) fraction (containing IncRNAs), (iv) 120–200nt RNAs (containing snRNAs and snoRNAs), and (v) 70–110nt RNAs (containing mostly tRNAs) (Fig. 2A). For each RNA pool, we quantified the m^{2,2}G levels using LC-MS/MS, in biological triplicates, and normalized its abundance to the abundance of unmodified bases in each RNA pool. As expected, we were able to detect m^{2,2}G in the 70–110nt RNA pool, which includes mature tRNAs. In addition, we also detected significant levels of m^{2,2}G in the 120–200nt RNA fraction (Fig. 2B), suggesting that m^{2,2}G is not only found in mature tRNA molecules. Similarly, we detected significant levels of m^{2,2}G in the 120–200nt RNA fraction in mouse brain samples (Fig. 2C), suggesting that m^{2,2}G is present beyond the mature tRNA fraction not only in neuronal cell lines, but also in tissues.

**LC-MS/MS identifies tRNAs as TRMT1 targets, but does not provide hints on TRMT1L targets**

To identify the biological targets of TRMT1 or TRMT1L, we analysed the m^{2,2}G levels in mouse TRMT1L knockout and human patient-derived TRMT1-deficient samples, compared to wild-type, using LC-MS/MS (Fig. 2C-D, see also Methods). The data show that TRMT1L is largely responsible for m^{2,2}G modification in tRNAs, in agreement with previous observations [31,32]. By contrast, TRMT1L knockout did not significantly alter the global m^{2,2}G modification levels of any of the 3 RNA fractions examined (Fig. 2C).

We then wondered whether TRMT1L might in fact be responsible for placing an RNA modification different to m^{2,2}G, which could explain why m^{2,2}G levels do not significantly vary between TRMT1L knockout and wild type samples. Using LC-MS/MS, we quantified the RNA modification levels of 27 distinct RNA modifications across 7 RNA pools of distinct RNA sizes (Figure S5A,B). We found that the depletion of TRMT1L led to modest decrease in the m^{2,2}G levels in the 120–200nt RNA fraction as well as in the m^{2,2}G levels in the 70–110nt RNA fraction (Figure S5A), although neither of these were found to be significant.

**Mapping of TRMT1L-dependent modifications in the brain transcriptome**

Whilst LC-MS/MS is a sensitive methodology that can accurately quantify RNA modifications, it does not identify which RNAs molecules are in fact modified. Therefore, we examined the RNA modification activity of TRMT1L using next-generation sequencing. Indeed, certain RNA modifications, such as m^{2,2}G, affect the Watson-Crick base pairing moity, and can be detected in the form of ‘mismatch signatures’ in RNA-seq datasets [36–39]. The dimethylation of guanosine at the N2 position eliminates the ability of the N2 to function as a hydrogen bond donor, altering its pairing behaviour, and
Figure 2. Quantification of m$^2$G in different RNA pools using LC-MS/MS. (A) RNA fractionation strategy used for LC-MS/MS samples. (B) LC-MS/MS m$^2$G quantification in the 5 different RNA pools of neuron-derived SH-SY5Y cells. (C) Relative proportion of m$^2$G in 3 size selected RNA pools in TRMT1L KO mice brain samples, relative to WT mice brain samples. Standard deviation in panels B C and D denotes biological triplicates, except in the positive and negative controls, where standard deviation denotes technical triplicates. E. coli total tRNA was used as negative control (C-), as it does not contain m$^2$G modifications, and S. cerevisiae tRNA$^{Ala}$ was used as positive control (C+), as it is known to contain m$^2$G in position 26. (D) Relative proportion of m$^2$G in 3 size selected RNA pools in human TRMT1 mutant patient-derived lymphoblastoid cells, relative to human TRMT1 wild-type patient-derived lymphoblastoid cells. Statistical significance was assessed using unpaired t-test (ns = non-significant change; * = p-value < 0.05).

consequently affects the misincorporation and/or drop-off rate at that given position when reverse transcribed [40]. Similarly, previous studies have shown that m$^2$G and m$^2$G modifications can be detected in the form of altered mismatch frequencies [41], although the latter only causes moderate misincorporation defects [40,42,43].

Overall, m$^2$G RNA modifications constitute strong candidates for being detected using the non-random mismatch signature strategy in RNA-seq datasets.

We performed both small and long RNA sequencing of mouse brain samples in both TRMT1L knockout and wild-type mice in biological triplicates (Fig. 3A), using a modified library preparation protocol to capture small RNAs up to 150 nt (see Methods). We found that our strategy was able to capture known m$^2$G modification sites in position 26 of multiple tRNAs, in a highly reproducible manner (Fig. 3B). We observed that the presence of m$^2$G modifications caused characteristic mismatch signatures at the modified sites, mainly in the form of G-to-T mismatches (Fig. 3C), in agreement with previous reports [41]. By contrast, we were unable to identify mismatch signatures in the vast majority of m$^2$G-modified sites in mouse tRNAs. Thus, we concluded that our RNA-seq mismatch analysis would be capturing information related to changes in m$^2$G modifications levels, but not from m$^2$G-modified sites.

We then proceeded with differential mismatch frequency analysis, first focusing on the subset of reads mapping to mature tRNAs (see Methods). Our analysis identified 3 tRNA$^{Ala}$(AGC) gene clusters whose mismatch error at position 26 completely disappeared in all 3 replicates, suggesting that m$^2$G modifications present in these tRNAs were exclusively placed by TRMT1L (Fig. 3D,E). We then examined the mismatch signature in reads mapping to other RNA pools, including small non-coding...
Figure 3. M$^{2,2}$G, but not m$^7$G, can be identified via non-random mismatch signatures in RNA-seq datasets. (A) Strategy for transcriptome-wide detection of m$^{2,2}$G RNA modifications. (B) IGV tracks illustrating that previously known m$^{2,2}$G modifications are detected in position 26 of eukaryotic tRNAs, both in wild type and knockout strains. Positions with mismatch frequency greater than 10% are coloured according to the relative base frequency at that position (A: green, G: orange; T:red, C: blue), otherwise the position is depicted in grey. (C) Ternary plot of the mismatch signatures of m$^{2,2}$G, m$^7$G and m$^7$G in known tRNA-modified positions. (D) IGV tracks of both WT and TRMT1L KO centred on several tRNA$^{AGC}$ gene clusters, showing that the mismatch signatures observed in position 26 consistently disappear in the knockout strain, in all 3 biological replicates. The m$^{2,2}$G26 sites are depicted with green asterisks. Positions with mismatch frequency greater than 10% are coloured according to the relative base frequency at that position (A: green, G: orange; T:red, C: blue), otherwise the position is depicted in grey. (E) Comparison of the mean mismatch frequencies observed in tRNA gene clusters of TRMT1L knockout brain samples, relative to wild type. Only tRNA positions with mismatch frequencies greater than 0.1 in the wild type strains have been included in the analysis. The 3 tRNA$^{AGC}$ gene clusters that show differential mismatch frequency in TRMT1L knockout samples are highlighted in red. Mismatches seen in positions 34 and 37 of tRNA$^{AGC}$ correspond to mismatch signatures caused by the presence of inosine (I34) and 1-methylinosine (m1I37), respectively.

RNAs, pre-tRNAs, tRNAs, lincRNAs and mRNAs; however, we did not identify any significant site whose mismatch frequency changed upon TRMT1L knockout (Figure S6).

**tRNA$^{Ala}$ (AGC) genes modified by TRMT1L are not exclusively expressed in neuronal tissues**

Previous studies have shown that a mutation in the central nervous system (CNS)-specific tRNA$^{Ala}$ (UCU) gene can lead to neurodegeneration in mice [44]. The fact that the tRNA$^{Ala}$ (UCU) gene is only expressed in neuronal tissues explains why the phenotype of the mutant mice was neurological. Following this line of thought, we reasoned the subset of tRNA$^{Ala}$ genes that is targeted by TRMT1L might be only expressed in neuronal tissues, thus potentially explaining the neurological phenotype observed in TRMT1L knockout mice.

To test this hypothesis, we re-analysed publicly available small RNA-seq data from mouse brain and liver samples across developmental stages [45]. Pairwise comparison of tRNA levels in brain and liver tissues identified two tRNAs that were brain-specific: tRNA$^{Ala}$ (UCU), as previously reported, and tRNA$^{Ala}$ (UGC) (Figure S7A). However, we did not identify any tRNA$^{Ala}$ (AGC) that was brain-specific (Figure S7B). Thus, we conclude that the cognitive impairment observed in TRMT1L knockout mice is not a simple consequence of brain-specific expression of the tRNA genes that are being modified by the enzyme.

**Neuronal activation causes subcellular relocalization of the m$^{2,2}$G modification machinery**

To explore the roles of TRMT1 and TRMT1L in neuronal activity, we compared SH-SY5Y neuroblastoma cells before and after depolarization with potassium chloride (KCl),
a system that has been used to mimic long-term potentiation (LTP) [34]. Adding 50 mM KCl to the medium for 30 seconds causes a short depolarization burst that leads to changes in the expression of immediate early gene (IEG) levels in neuronal cells [46], which have been used as markers for LTP in neuronal cell lines [47]. Stimulation-dependent changes in gene expression of the IEGs (C-FOS, ARC, EGR1) were validated by qPCR (Figure S8). Under these conditions, we found that TRM1L relocates from the cytoplasm and mitochondria to the nucleus (Fig. 4A). Likewise, TRMT1L moves from the nucleoli to a similar dotted domain pattern in the nucleus (Fig. 4B,C). A co-localization experiment showed that, even though TRMT1 and TRMT1L have a similar pattern after activation (Fig. 4D), they do not colocalize in the nucleus (Fig. 4E), suggesting that they are being trafficked to different specialized subnuclear domains whose function(s) are yet unknown, indicating the precision and importance of dynamic subcellular localization, the mechanisms of which are also unknown. Moreover, it suggests that these two enzymes may have different RNA targets both before and after activation.

We then asked whether the relocation of TRMT1 to the nucleus upon neuronal activation might be accompanied by a change in the m^2G levels of the cells. To this end, we analysed the m^2G levels of each small RNA fraction individually (120–200nt, 70–110nt and 20–50nt) in SH-SY5Y cells during activation (t = 0 h, 6 h and 24 h post-activation). Although we observed a slight decrease in m^2G levels upon neuronal activation, these differences were not statistically significant (Fig. 4F).

Previous studies have shown that the activity of m^6A RNA writer and reader enzymes, as well as the RNA modification levels, vary upon exposure of cells to stress conditions, such as heat shock or chemical stress [48–50]. Thus, we investigated whether exposure to heat shock might also cause changes in the localization of TRMT1 and TRMT1L. We found that neither TRMT1 nor TRMT1L change their localization patterns upon exposure to heat shock stress (Figure S9) during a 24 h time course, in contrast to our observations when exposing SH-SY5Y cells to neuronal stimulation conditions (Fig. 4A,B). Altogether, our results suggest that the relocalization of TRMT1 and TRMT1L is not a general response to cellular stress, but rather a specific phenotype related to neuronal activation.

**Discussion**

Base modifications overlay sequence information to expand the lexicon of RNA, introducing changes into structural, coding or regulatory information. RNA modifications are involved in many biological processes, including cellular differentiation [51], sex determination [52,53], maternal-to-zygotic mRNA clearance [54] and stress responses [48,55]. However, much less is currently known about the roles of RNA modification in synaptic plasticity and or memory formation [2]. In this regard, several works have provided genetic evidence that some of these RNA modifications, including ribose, cytosine and guanine methylation, may play a role in cognitive function [25–30]. Unfortunately, with the exception of m^6A modifications [21,56–58], the dynamics of RNA modification in neuronal biology have yet to be studied, largely due to the difficulty of identifying RNA modifications, a problem that is beginning to be solved by the use of direct RNA sequencing nanopore technologies [59–62].

In this study, we focused on two RNA methyltransferases that have been previously associated with cognitive dysfunctions: TRMT1, the loss of which causes intellectual disability in humans [27], and its ortholog TRMT1L, the loss of which causes behavioural changes and motor defects in mice [33]. We confirmed that TRMT1 is responsible for placing m^2G in tRNA molecules (Fig. 2D), and revealed that TRMT1L is responsible for placing m^2G in a subset of tRNA^Ala/(AGC) genes (Fig. 3D,E). Perhaps not coincidentally, a number of neurological defects result from the loss of tRNAs and related enzymes, whose expression is not necessarily brain-specific [10,13,63,64] and whose effects may be mediated through products of tRNA processing [65].

Our LC-MS/MS results suggest that m^2G is present not only in tRNAs, but also in the RNA fraction of 120–200 nt (Fig. 2B, see also Figure S5A). To our knowledge, this is the first time that m^2G is observed beyond the tRNA fraction. Depletion of TRMT1 activity did not alter the m^2G levels detected in this RNA fraction, suggesting that TRMT1 is not the enzyme responsible for placing m^2G in this RNA pool. By contrast, we observed a modest -yet not statistically significant- decrease in the m^2G levels present in the 120–200 nt fraction upon TRMT1L depletion. Unfortunately, our bioinformatic pipeline used to analyse the RNAseq libraries from TRMT1L wild type and knockout mice brain samples did not identify any m^2G modifications present in RNA molecules beyond tRNAs (Figure S6). One possibility is that this specific RNA pool is in fact not modified by TRMT1L, and that an additional enzyme, evolutionarily unrelated to the TRM domain, might be placing m^2G modifications in the 120–200 nt RNA fraction. Alternatively, the m^2G modification stoichiometries in these RNAs might be low, which could explain why our bioinformatic pipeline to identify m^2G modifications using mismatch signatures might have missed it, as they can only capture sites that are modified at mid-high stoichiometries. Future work will be needed to identify which RNA molecules -beyond tRNAs- contain m^2G modifications, as well as to reveal which is the enzyme responsible for placing them.

Previous works have shown that nuclear bodies, including nucleoli, Cajal bodies, nuclear speckles and paraspeckles can dynamically respond to stress, altered metabolic conditions and alterations in cellular signalling [66]. Here we show that TRMT1 and TRMT1L are localized in different subcompartments in the cell (Fig. 1) and that their localization, and presumably their targets, are altered upon neuronal activation (Fig. 4). To the best of our knowledge this is the first time that the localization of RNA modification enzymes has been shown to change in response to neuronal activation. The subcellular and subnuclear compartments between which they shuttle (with the exception of the nucleolus) are as yet largely uncharacterized, indicating another unexplored level of neuronal organization, embedded in domains that
are likely scaffolded and potentially phase-separated by other RNAs [67,68]. This level of complexity is unprecedented and opens new perspectives in the complex path to the understanding of brain development and neuronal function.

A plethora of molecular functions for RNA modifications have been revealed in the past few years, including the regulation of alternative splicing, translation efficiency and mRNA half-life [69–72]. Although it is yet unclear to what extent RNA modifications may affect the subcellular localization of

Figure 4. Neuronal activation causes TRMT1 and TRMT1L subcellular re-localization and affects m$^2$G cellular levels. (A, B) Immunofluorescence experiments of SH-SYSY cells upon neuronal activation, imaged at 0 h (untreated, T0), 1 hour post-activation (T1), 3 hours post-activation (T3) and 6 hours post-activation (T6). SH-SYSY cells were stained with anti-TRMT1 (A) or anti-TRMT1L (B) antibodies. DAPI was used as a nuclear marker. (C) Schematic illustration of the relocation patterns that occur upon neuronal activation, for TRMT1 (left) and TRMT1L (right). (D) The proportion of cells stained in the nucleus (upper panel) upon neuronal activation. In the bottom panel, the proportion of cells with TRMT1L nuclear dotted pattern upon activation is shown. (E) Immunofluorescence of SH-SYSY cells before (T0) and 6 hours after neuronal activation (T6). Upon activation, TRMT1 relocates from cytosol and mitochondrial to the nucleus, but does not colocalize with TRMT1L. (F) Abundance of m$^2$G RNA modifications in different RNA pools upon neuronal activation, measured at three time points (t = 0 h, 6 h and 24 h post-activation). E. coli total tRNA was used as negative control (C−), and yeast tRNA$^{Phe}$ was used as positive control (C+). Error bars represent standard deviations from 3 biological triplicates. Statistical significance was assessed using unpaired t-test (ns = non-significant change; * = p-value < 0.05).
RNAs, previous works have shown that some RNA modifications facilitate phase separation [68,73] and have proposed their involvement in RNA relocalization in neuronal cells [57,74]. Although evidence for a widespread involvement of RNA modifications in RNA localization is somewhat limited, observations across diverse systems and organisms urge for additional investigation. Future work will be needed to elucidate what is the interplay between modified RNAs, their modifying enzymes and the RNA subcellular localization in neuronal systems, as well as to characterize the functional consequences of the relocalization of RNA molecules to specific subcellular compartments.

Materials and methods

Cell culture and passaging

Human SH-SY5Y neuroblastoma cell lines were cultured in Gibco Life Technologies Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% Penicillin Streptomycin (Life Technologies, #15070-063). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were grown until they reached 80% confluency and washed once with PBS before splitting. For a 150 cm² flask, cells were incubated with 5 ml trypsin (Sigma), for 2 min at 37°C for detachment. Trypsin was neutralized by adding 15 ml of DMEM with 10% FBS. From this mix, 5 ml was added to 30 ml of DMEM with 10% FBS in a new flask of 150 cm², bringing the cells to a confluence of 20%. Cells were discarded when they reached passage number 12.

Patient-derived TRMT1 mutant and control lymphoblastoid cell lines

B-lymphoid cell lines (LCLs) were established from healthy and affected individuals (Figure S10) by in vitro infection of peripheral blood mononuclear B cells with Epstein Barr Virus (EBV) based on standard protocols [75]. The mutations in the TRMT1 gene (NM_017722) in the patients used to derive the LCLs, as well as the clinical phenotypes of these patients have been previously described [27,76]. Healthy individuals used in this work contained a single copy of the mutated TRMT1 gene and did not show intellectual disability or apparent clinical phenotypes [76], whereas affected individuals contained both TRMT1 gene copies with the 1332_1333 deletion in the coding sequence of TRMT1, and showed intellectual disability (ID) as well as other clinical phenotypes (Table S1). All LCL cell lines used in this study were taken from a family with a pY445fs mutation in the TRMT1 coding region (c.1332_1333delGT; p.Y445fs). TRMT1 pY445fs mutation was also confirmed in the LCLs through RNAseq analysis (Figure S11). To generate LCLs, 10 ml of peripheral blood was collected from each patient in a sterile preservative-free heparin tube. Mononuclear cells were isolated from whole blood by density Ficoll-Hypaque gradient centrifugation, washed with medium RPMI three times. Pellets were resuspended in transformation-medium (B95-8 virus suspension, RPMI 1640, 20% FBS, 0.2 µg/ml Cyclosporin A). After obtaining appropriate growth, cells were transferred in a 25 cm² filtered flask, and cultured in RPMI 1640 (Gibco) supplemented with 1% penicillin-streptomycin (Gibco), 15% fetal bovine serum (FBS) (Gibco), 1 mM L-glutamine, 10 mM HEPES and 1% Gibco™ AmnioMAX™ in a 5% CO₂, humidified, 37°C incubator up to 3–4 weeks. The culture media were changed every 3–4 days when cells showed extensive growth. Total cellular RNA was isolated from LCLs using VIOGENE miTotal RNA extraction mini-prep kit (Cat #VTR1002), in biological triplicates.

TRMT1L knockout mice brain RNA samples

Trmt1-like KO mice have been previously generated and described [33]. Briefly, ES cells with a gene trap vector integration in intron 1 of the mouse Trmt1-like gene, were used to generate chimeric mice by morula aggregation with wildtype embryos (E2.5) obtained from superovulated CD1-females (Charles River). Following overnight culture of aggregates, blastocysts were transferred to foster mothers and chimeric offspring were mated to C57BL/6 (Charles River) mice to generate heterozygous Trmt1-like progeny. Heterozygote mice were continuously backcrossed to the C57BL/6 line for more than 10 generations and characterized [33]. Tissue samples for this study were obtained from Trmt1-like WT/WT and Trmt1-like GT/GT mutant mice at the same age of 96–97 days. Mice were culled by cervical dislocation and brains were immediately dissected and snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was obtained by homogenizing whole brain tissues from wildtypes and Trmt1-like mutants using POLYTRON PT 1200E followed by Trizol extraction. TRMT1L knock-out was also confirmed through RNAsseq analysis (Figure S11).

Plasmids for over-expression of TRMT1-FLAG and TRMT1L-FLAG

TRMT1 and TRMT1L CDS sequences were cloned from the MGC premier human ORFeome v8.1 library. The TRMT1-FLAG construct consisted of the CDS of TRMT1 (Human ORFeome, Internal ID: 378, Clone ID: 3463518) followed by a FLAG epitope (DYKDDDDK) at the C-terminal end, whereas the FLAG-TRMT1L construct consisted of the CDS of TRMT1L (Human ORFeome, Internal ID: 10729, Clone ID: 5296208) with the addition of a FLAG epitope (DYKDDDDK) at the N-terminal end. FLAG-tagged constructs were inserted into a pcDNA3.1+ plasmid, respectively, and were used in subsequent transfection experiments. The FASTA sequences of the TRMT1-FLAG and TRMT1L-FLAG constructs used in this work can be found in Table S3.

Transfection and transient expression of TRMT1-FLAG and TRMT1L-FLAG

HEK293 cells were plated on glass coverslips in 12-well plates at 60.000 cells per coverslip and left overnight to adhere to the glass. Each well was transfected with 200 µl of serum-free
medium, 4 µl of lipofectamine 2000 reagent and 1.6 µg of plasmid according to the manufacturer’s instructions. Cells were then transfected with either FLAG-tagged versions of TRMT1 (TRMT1-FLAG) or TRMT1L (TRMT1L-FLAG). Cells on coverslips were fixed 24 h after transfection and used for immunofluorescence assays.

**Immunofluorescence assays**

Coverslips were placed in 24-well plates, coated with Poly-L-Lysine for 5 min and dried overnight after washing 4 times with water. SH-SY5Y cells were plated at a density of 2.5 × 10^5 in 500 µl of DMEM with 10% FBS and grown overnight. Cells were washed once at room temperature with PBS and fixed in pre-warmed (37°C) 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) at room temperature for 15 min and then washed 3 times for 5 min with PBS. Cells were then permeabilized in 0.1% Triton X-100/0.1% Na-Citrate/PBS for 3 min at room temperature, and washed 3 times for 5 min in PBS. Cells were blocked in 10% FBS/PBS for 1 h at room temperature and incubated with anti-TRMT1 primary antibodies (dilution 1:100, Ab134965) or anti-TRMT1L (dilution 1:200, NBP18337) in blocking solution overnight at 4°C. Cells were then washed 3 times for 5 min in PBS, and incubated with secondary antibodies for 90 min at room temperature, followed by 4 washes with PBS. Coverslips were dipped in demineralized water, and mounted using immuno-fluore mounting medium (MP Biomedicals). Mitochondria staining was achieved using MitoTracker Red FM (Thermo Fisher), by adding MitoTracker 45 min before fixation to the growing medium to a final concentration of 100 nM. DAPI was employed at a 1:100 dilution, whereas phalloidin was employed at a 1:500 dilution. For co-immunofluorescence experiments of FLAG-tagged versions of TRMT1 and TRMT1L, coverslips were washed twice in PBS prior to fixation with 4% PFA for 20 minutes at room temperature, then washed twice with PBS. Cells were then permeabiled with 0.5% TritonX-100 in PBS for 15 minutes at room temperature, washed with PBS-T 0.1% and then blocked with 5% FBS in PBS-T 0.1%. Cells were then stained with rabbit anti-TRMT1 primary antibody (dilution 1:100, Ab134965), rabbit anti-TRMT1L (dilution 1:200, NBP18337) and/or mouse anti-FLAG (Sigma F1804-50UG, dilution 1:500) in blocking solution overnight at 4°C. Cells were then washed 3 times for 10 minutes in PBS-T 0.1%, and incubated with secondary antibodies Alexa Fluor 488 anti-mouse (Thermo Fisher Scientific A-11001, dilution 1:2000) and/or Alexa Fluor 555 anti-rabbit (Thermo Fisher Scientific A-21429, dilution 1:2000) in PBS-T 0.1% with Hoechst (1 µg/ml) for 90 minutes at room temperature, followed by 3 washes with PBS-T 0.1%, then coverslips were let dry and mounted.

**Confocal imaging and image analysis**

Confocal images were acquired using a LSM700 confocal laser-scanning upright microscope (Zeiss) with a 63x oil objective. The zoom was set between 1x and 2x, with a pinhole of 34 µM and a speed of 1.58 µs per pixel. Confocal laser intensity was set to 3 and the gain was adjusted per sample. The dimensions were set to 1024x1024pixels and averaged 4 times. All experiments were repeated 3 times and for each condition. The cell counter plugin of Fiji 1.49 (ImageJ) was used to quantify the movement of TRMT1 and TRMT1L after cell activation with KCl. Statistical analysis was done using GraphPad Prism 6, using linear regression analysis. Images for transient expression of FLAG-TRMT1 and FLAG-TRMT1L were acquired on a Leica-SPE microscope with a 63x oil objective and processed with Fiji 1.52.

**Cellular fractionation**

Cell fractionation was performed according to the protocol described by Suzuki et al [77], with minor modifications. SH-SY5Y cells were grown as monolayers in 15 cm diameter dishes until reaching 80% confluence. Cells were washed twice in ice-cold phosphate buffer saline (PBS) pH 7.4 (130 mM NaCl, 2 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4), scraped from culture dishes and collected in 1.5 mL of ice-cold PBS. After 15 sec centrifugation in a top table microcentrifuge (Thermo Scientific, USA), cell pellets were resuspended in 750 µl of ice-cold 0.05% Nonidet P-40 (Roche Diagnostics, Germany) in PBS and 200 µl of the lysate was removed as ‘whole cell lysate’. The remaining material was centrifuged for 15 sec and 300 µl of the supernatant was removed as the ‘cytoplasmic fraction’. After removal of the remaining supernatant, the pellet was resuspended in 750 µl of ice-cold 0.075% NP40 in PBS, centrifuged for 15 sec and the supernatant was discarded. The pellet was designated as the nuclear fraction. Whole cell lysates and cytoplasmic fractions were quantified by measuring OD 280 nm in the Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

**Western Blot**

Following subcellular fractionation, SH-SY5Y were washed with PBS, harvested and lysed with 70 µl of RIPA buffer. Samples were then sonicated for 7 sec to fragment the DNA for easier loading. LDS Sample Buffer (Novex #B0007) was added and samples were heated to 70°C for 15 min. 20 µl were loaded onto a Novex Bolt 4–12% Bis-Tris Plus gel, and separated by electrophoresis for 50 min at 200 V. Following electrophoresis, samples were transferred to PVDF membranes using wet transfer apparatus (Xcell Surelock, Invitrogen) at 30 V for 90 min. Membranes were blocked in 5% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature. Membranes were incubated with anti-TRMT1 (1:10,000, Ab134965) or anti-TRMT1L (1:2,000, NBP-1 88337) antibodies at 4°C overnight with rotation. After washing with TBS-T 3 times for 5 min, membranes were incubated for 90 min with appropriate horseradish peroxidase-conjugated secondary antibodies at 37°C. Protein bands were visualized using SuperSignal West Pico chemiluminescent Substrate (Thermo #34080) and imaged using Fusion FX (Vilber). Normalization and intensities were analysed using FusionCapt Advance FX7. For loading controls values were normalized against GAPDH (dilution 1:50,000, Ab8245) and Histone H3 (dilution 1:50,000, Ab1791).
RT-qPCR

SH-SY5Y cells plated in 6-well plates were lysed using 750 μl of QIAzol Lysis reagent (Qiagen) according to manufacturer’s instructions. Total RNA was extracted using the RNasy mini kit (Qiagen #74104) according to manufacturer’s instructions. First strand cDNA was synthesized from 1 μg RNA, using SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies), and the synthesized cDNA was stored at −20°C until use. RT-qPCR was performed using the Bio-Rad CFX384 Real-Time PCR Detection System using primers (Table S2), using GAPDH and PGK1 as internal controls. During the extension phase the fluorescent signals were collected and Ct values of the samples were calculated. Transcription levels of TRMT1 and TRMT1L were normalized to controls by the ∆∆Ct method (Livak and Schmittgen 2001). Results from triplicate experiments were grouped and an unpaired t-test was used to compare groups.

Preparation of samples for mass spectrometry: RNA isolation

For extracting different fractions of RNA from SH-SY5Y cell cultures, cells were grown until 80% confluency in a 150 cm² flask. Cells were then washed once with PBS, incubated with 5 ml Trizol at room temperature for 5 min, and collected using a cell scraper. For each ml of sample, after which 300 μl of chloroform were added, vortexed for 15 s, incubated 5 min at RT, and spun down for 15 min at 4°C. The clear top phase was moved to a new tube, and the miRNAeasy kit (Qiagen #217004) was used to separate the RNA contained in this phase in two fractions: <200 nt and >200 nt. Briefly, 1VOL of 70% ethanol was added to the RNA phase and vortexed for 5 s. The sample was then transferred to a miRNAeasy spin column and spun down for 30 s at max speed. This column collects all RNAs larger than 200 nt. 0.65 VOL 100% ethanol was added to the flow-through of the first spin column, and vortexed. Mix was then added to a second spin column to capture RNAs smaller than 200 nt. Both columns were washed with 700 μl RWT, 500 μl RPE (2x), and 80% Ethanol. Columns were centrifuged and air-dried before adding 30 μl RNAse free H2O.

Preparation of samples for mass spectrometry: RNA fractionation

The >200 nt RNA fraction was ribo-depleted using non-overlapping DNA oligonucleotides that are complementary to rRNA 18s and 28s followed by digestion with Hybridase™ Thermostable RNaseH (Lucigen #H39500), as previously described [78]. Briefly, 10 μg RNA sample was ribo-depleted by adding 10 μl of rRNA oligo library (100 μM), and incubated with 1x Hybridization bufer to a total volume of 20 μl. The incubation consisted in a down ramp from 95°C to 45°C with −0.1°C/s. When 45°C was reached, RNaseH (2 U/μl), MgCl2 (20 mM) was added and final volume was brought to 40 μl, and incubated at 45°C for 30 min. After incubation, 10 μl of H2O was added to bring the final volume to 50 μl. Then, 90 μl of RNAclean XP beads with 270 μl 100% Isopropanol was added for the purification step. Samples were placed on a magnet for 5 min and washed twice with 300 μl 85% Ethanol. Samples were then air-dried and RNA was eluted in 30 μl RNase free H2O. After ribodepletion, Dynabeads Oligo(dT)25 (Invitrogen #61002) were used to separate the mRNA fraction. Dynabeads were resuspended thoroughly and placed on a magnet to aspirate the supernatant, and washed with 100 μl of Binding Buffer (20 mM Tris-HCl, pH7.5, 1.0 M LiCl, 2 mM EDTA) twice. After washing, Dynabeads were resuspended in 100 μl Binding Buffer. Samples were then adjusted to 75 μg in 100 μl 10 mM Tris-HCl pH 7.5, RNA-free H2O. 100 μl of Binding buffer was added to the samples, and were then incubated at 65°C for 2 min to disrupt secondary structures and immediately placed on ice. Then 100 μl of washed beads were added and the mix was placed on a rotator for 5 min. After rotation, samples were placed on a magnet for 2 min and supernatant containing lncRNA was removed and stored on ice. Beads were resuspended in Washing Buffer B by pipetting carefully. Supernatant was again removed on the magnet and this step was repeated once. mRNA was then eluted from the beads with 20 μl 10 mM Tris-HCl by heating to 80°C for 2 min. The samples were then immediately placed on the magnet and eluted mRNA was transferred to a new tube and stored in −80°C. The supernatant containing the lncRNA was precipitated by adding 0.1x the volume in Ammonium Acetate 7.5 M and 3x the volume in 100% Ethanol with 1 μl of Pellet paint. Samples were vortexed for 10 s and incubated overnight at −20°C. The next day samples were spun down for 30 min at 4°C. Pellet was washed twice with 75% Ethanol and air-dried for 15 min before eluting in 30 μl RNAse-free water.

The <200nt RNA fractions were treated with Turbo DNase (Invitrogen AM2238) according to manufacturer recommendations. After the treatment, RNA samples were recovered on column using the kit RNA Clean & Concentrator (Zymo Research R1017). Samples were further subdivided into three fractions (120–200nt, 70–110nt, and 20–50nt) by electrophoretic separation and gel excision. Briefly, a 15% TBE-Urea gel (Invitrogen, 1.0 mm x 10wells, #EC6885BOX) was pre-run at 100 V for 30 min with 0.5x TBE (Novex TBE running buffer, #LC6675). Approximately 8 μg of <200nt RNA per sample was mixed to an equal volume of 2x loading dye (NEB #B0363A) and incubated at 70°C for 5 min. The gel wells were washed from urea, and 20 μl per well was added, leaving an empty well between samples to avoid cross-sample contamination. Electrophoresis was carried out at 100 V for 2 hours. The gel was then placed on a shaker for 5 min in 0.5x TBE, RNAse free H2O with 1:10,000 SYBR Gold nucleic acid gel stain (Life tech #S11494), and three bands were excised under a UV-light. RNA was extracted from the gel and purified using the kit ZR small-RNA PAGE Recovery (Zymo Research R1070) following the manufacturer recommendations. Samples were eluted from the column in 10 μl RNAse-free water.

Digestion of RNA into nucleosides for LC-MS/MS

For RNA nucleoside digestion, a maximum of 1 μg of sample was mixed with 250 U Benzonase Nuclease (E1014-5KU), 200
mU Phosphodiesterase I (P-3243, Sigma), 200 U Alkaline phosphatase (P-7922-2KU, Sigma), 20 mM Tris-HCl buffer pH 7.9, 100 mM NaCl, 20 mM, and H₂O up to 50 µl, and incubated for 6 hours at 37°C. After incubation samples were stored in −80°C until the LC-MS/MS experiment. tRNA₃⁵⁺ from S. cerevisiae (Sigma, R4018) was used as positive control (m²⁺G-positive), and total tRNA from E. coli (Sigma, R1753) was used as negative control (m⁻²G-negative).

**LC-MS/MS analysis – m22G modifications**

The UHPLC-MS/MS system consisted of a Ultra High Performance Liquid Chromatography Acela Pump (Thermo Fisher Scientific, Waltham, USA) and HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled directly to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Fisher Scientific) via an electrospray interface. Liquid chromatography was performed on an Acuity UHPLC HSS T3 Column, 2.1 × 100 mm, 1.8 µm (Waters, Milford, USA). 10 µl of the sample was analysed using gradient elution with aqueous 0.1% formic acid (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow of 0.4 ml/min over 7 minutes. The acetonitrile gradient increased linearly from 0% at 1.5 minutes to 100% at 6 minutes and held for 0.5 minutes before returning to 0% at 6.60 and remaining there until 8 minutes. The divert valve was set to waste during the first 0.6 minutes of the run. Analysis was performed in positive ionization mode using selected reaction monitoring (SRM). Mass spectrometric conditions were optimized for sensitivity using infusions of analyte standards. The sheath gas pressure and the auxiliary gas pressure were 30 and 5 (Thermo Fisher arbitrary units) respectively. The probe temperature was 200°C, the capillary temperature was 330°C and the tube lens offset was 90 V. The mass to charge transitions and collision energies used to detect and quantify the analytes monitored were, for adenosine (A): 268 (136 at 17 V), cytidine (C): 244 (112 at 12 V), guanosine (G): 284 (152 at 14 V), uridine (U): 245 (113 at 10 V), and d-(dimethylamino)guanosine (m²⁻G): 312 (180 at 15 V). 1 alternate SRM transition was also acquired for each analyte as a qualifier to assist if required in the case of interference. Standards used for calibration curves included: adenosine (Sigma-Aldrich, A9251), cytidine (Sigma-Aldrich, C122106), guanosine (Sigma-Aldrich, G6752), uridine (Sigma-Aldrich, U3750), 2-(dimethylamino)guanosine (Santa Cruz, sc-220667). Data processing of chromatograms was performed using the Quanbrowser function of the Xcalibur Software package version 2.0.7 (Thermo Fisher Scientific). Quantification was performed using an external calibration curve over the range 10 pg to 200 ng for A, C, G, U and 10 pg to 40 ng for m²⁻G. Samples were analysed in duplicate and the mean reported. Calibration curves were run at the start of each batch and then after approximately every 30 samples. The procedure described above applies to results shown in Figures 2 and Figures 4.

**Activation of SH-SYSY cells via KCl treatment**

Cells were plated on sterilized coverslips in 24-well plates for imaging at a density of 3 × 10⁵ cells, or in 6-well plates for RT-qPCR. Cells were activated at 60% confluence by adding 50 mM KCl to the medium. After 30 seconds, the medium was replaced with fresh medium. Cells were harvested and fixed at 0, 1, 3 and 6-hours post-activation. SH-SYSY cell activation was validated using RT-qPCR, which confirmed the upregulation in the expression of immediate-early genes (Hoffman et al. 1993; Okuno 2011; Minatohara et al. 2015).

**Heat stress exposure of SH-SYSY cells**

SH-SYSY cells were plated on sterilized coverslips in 24-well plates for imaging at a density of 3 × 10⁵ cells. Once they reached 60% confluence, the cells were exposed to 42°C for 20 minutes, which is considered a mild heat stress [80]. Cells were harvested and fixed at 0, 2, 6 and 24-hours post-heat exposure, and subsequently used for immunofluorescence assays.
Small and long RNA-seq library preparation and sequencing from mouse brains

Total RNA was extracted from TRMT1L KO and wild-type brain samples using TriZol. Both wild-type of TRMT1L KO brain mice were prepared for TruSeq Total RNA Illumina sequencing, in biological triplicates, for both the small (<200 nt) and large RNA fractions (>200 nt). Briefly, first-strand cDNA was synthesized using SuperScript II Reverse Transcriptase. RNA template was then removed, and second cDNA strand was synthesized using Second Strand Marketing MasterMix from the Illumina kit. cDNA was washed using AMPPure-Xp beads, and 3’ends were adenylated with A-Tailing Mix from the Illumina kit. Adapters containing Illumina barcodes for multiplexing were ligated to the end of the double stranded cDNA, and cDNA was washed and purified using AMPPure beads. DNA fragments containing adapters on both sides were amplified using 15 cycles of PCR. Size and purity of the sample was analysed on the Agilent 2100 Bioanalyzer. Sequencing was performed using the Illumina HiSeq 2500 platform with 125bp paired-end sequencing in the case of long RNA-seq datasets (>200 nt), and with 100 bp single-end reads in the case of small RNA-seq datasets (<200 nt).

Bioinformatic analysis of small RNAseq libraries

Small RNA-seq reads were processed with a mapping pipeline adapted from a previous tRNA mapping pipeline [81], with minor changes. The adapted version of the pipeline is publicly available in GitHub (https://github.com/novoalab/tRNAmap_Hoffmann_adapted). Briefly, reads were trimmed with bbduk (from bbmap 36.14) keeping reads with length 15–100 nt. Trimmed reads were then mapped with Segemehl 0.2.0–418 [82] to a modified mm10 genome complemented with tRNA genes and pseudogenes masked, and pre-tRNA genes appended as additional chromosomes. Segemehl options of this first mapping were: accuracy = 80, differences = 3. Reads mapping exclusively to the pre-tRNA reference (not mapping to other genomic sites) were kept for mapping to unique sequences of mature tRNAs (downloaded from gTRNAdb http://gtrnadb.ucsc.edu/ncrnadb 10/ on October 2019). This second mapping was performed with Segemehl with accuracy = 85 and differences = 3. Multimapping reads were kept only if they were showing the same mismatch profile with the different mapping loci (‘phased’ multimapping handling), as done in the original tRNA mapping pipeline [81]. In addition to tRNA mapping, we also analysed the mismatch signatures of other ncRNAs. For the ncRNA analysis, the trimmed reads were mapped with Segemehl 0.2.0–418 to a custom reference of selected types of ncRNAs (miRNA, scRNA, snoRNA and snRNA) downloaded from BioMart (GRCm38.p6) with default options, accuracy = 95 and differences = 0.

Bioinformatic analysis of long RNAseq libraries

125 bp paired-end long RNAseq reads were processed with Cutadapt v.1.9.1 [83] with (adapter sequence: AGATCGGAAGAG, with -m 1 option to exclude empty reads). Reads were aligned with STAR [84] version 2.7.0 f with default parameters to mouse genome (GRCm38) with vM21 genencode annotation. Long RNAseq reads were also mapped to mouse canonical tRNA sequences using Bowtie2 (local mode, default settings, -N 1).

Detection of m$^{2,2}$G modifications using mismatch signatures

Modified positions were identified for both small and long RNA mapped reads through the generation of mpileup files using the Samtools mpileup function [85]. Mpileup files were further processed with an in-house script (https://github.com/novoalab/mpileup2stats), which generates frequency tables of mismatches, insertions, deletions and RT-drop offs using mpileup files as input. Only positions with a minimum coverage of 10 reads/base were considered for downstream analysis. m$^{2,2}$G candidate sites were identified as those with a mismatch frequency difference (WT-KO) greater than 25%.

Bioinformatic analysis of TRMT1 and TRMT1L sequences

Protein fasta sequences from TRMT1 and TRMT1L proteins were obtained from the Uniprot [86] database (https://www.uniprot.org). Mitochondrial targeting peptides were predicted using MitoProt II [87] version 1.101 (http://ihg.gsf.de/ihg/mitoprot.html). Predictions of Zinc finger annotations were extracted from the PFAM [88] database (http://pfam.xfam.org/). Nuclear localization signals (NLS) were predicted using the SeqNLS [89] software (http://mleg.cse.sc.edu/seqNLS/). SeqNLS predicted TRMT1L to have a strong NLS signal (NLS_score ≥ 0.9) and TRMT1 to have weak NLS signal (0.5 < NLS_score < 0.85).

Phylogenetic tree building and homology searches

Complete annotated and curated reference proteomes were downloaded from Uniprot (www.uniprot.org/). Selecting only one strain per species, we obtained a final dataset of 1,597 complete proteomes, which included 119 Archaea, 1143 Bacteria, and 335 Eukarya. To identify all candidate m$^{2,2}$G methyltransferase enzymes, as well as closely related homolog domains, the hidden Markov Model (HMM) profile corresponding to m$^{2,2}$G methyltransferase catalytic domain (PF02005.15, TRM) was retrieved from the PFAM [88] database (https://pfam.xfam.org/). The TRM HMM profile was then used to query all Uniprot proteomes using the hmsmsearch function from HMMER [90] software v.3.2.1 (http://hmm.org/). Proteins above the default threshold were kept as candidate TRM proteins, and were aligned to the HMM profile using the hmmalign function in HMMER. This multiple sequence alignment was used as input to construct a maximum likelihood phylogenetic tree using PhyML [91].

Ethical statement

Ethical approval for the patient-derived samples from TRMT1 mutant was waived by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran
fractionation experiments and Mass Spectrometry sample preparation. HL and GL helped with the initial stages of bioinformatic data analysis. RP performed the Mass Spectrometry sample processing on the Quantum Access. DK prepared the next-generation sequencing libraries. GRF performed the subcellular fractionation and Western Blots in SH-SYSY cells. FY bred the mice from TRMT1L knockout and control groups, and collected brain tissues that were used for RNAseq and LC-MS/MS analysis. NC, SSA, HN and LRP cultured patient-derived TRMT1 wild-type and mutant lymphoblastoid cell lines and extracted total RNA for Mass Spectrometry and RNA-seq analyses. NS, JSM and EMN conceived the project. JSM and EMN supervised the project, with the contribution of DC. NJ, SC and EMN built the figures. NJ, SC, JSM and EMN wrote the paper, with the contribution from all authors.

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