HENMT1 restricts endogenous retrovirus activity by methylation of 3'-tRNA fragments

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ABSTRACT

Long terminal repeat (LTR) retroelements such as endogenous retroviruses (ERVs) utilize host tRNA as a primer for reverse transcription, and are thus susceptible to silencing by small RNAs derived from the 3′-end of mature tRNAs (3′-tRFs). Rigorous quantification reveals that tRNA level are not directly proportional to 3'-tRFs but specific isoacceptor and isodecoder sequences are highly enriched in a pattern conserved between mouse and human. We found 3'-tRFs are 2'-O methylated by the small RNA methyltransferase HENMT1 protecting them from degradation and promoting ERV silencing. In the absence of HENMT1, 3'-tRFs are subjected to non-templated tailing by the terminal nucleotidyltransferases TUT4 and TENT2 that regulate small RNA turnover. Due to the perfect sequence complementarity of 3'-tRFs to endogenous retroviral sequences, they have thousands of targets in mammalian genomes. We conducted a massively parallel reporter assay using *Mus musculus particle type D*, a highly active murine ERV, to determine target site rules for 3'-tRFs. Our results suggest that HENMT1 not only stabilizes germline integrity but also serves transposon control by 3'-tRFs in the soma.

INTRODUCTION

Transposable elements (TEs) are a threat to genome stability. They are usually embedded in inactive chromatin, but epigenetic reprogramming during development and disease erases repressive chromatin marks. This is when small RNA-mediated silencing mechanisms become crucial to regulate their expression, restrict their mobility and maintain genome integrity. In contrast to miRNAs in animals which function through partial complementarity, small RNAs that detect TEs usually recognize their target by near-perfect sequence complementarity and are stabilized by 2'-O methylation at their 3'-end (Ji and Chen, 2012). This modification is mediated by HENMT1 and its homologs across eukaryotes, and defects in methylation generally lead to destabilization of small RNA substrates (Ji and Chen, 2012; Park et al., 2002). In animals, HENMT1 is known for its role in piRNA biogenesis in the germline. Animal miRNA which are not methylated and piRNA that lost 2'-O methylation are prone to untemplated nucleotide addition to their 3'-ends by terminal nucleotidyltransferases, often preceding degradation (Gainetdinov et al., 2020; Kamminga et al., 2010; Yang et al., 2022; Yu and Kim, 2020).

Small RNAs derived from the 3'-end of tRNAs (3′-tRFs) inhibit long terminal repeat (LTR)-retroelements by targeting their highly conserved tRNA primer binding site (PBS) (Li et al., 2012; Schorn et al., 2017; Yeung et al., 2009). The use of host tRNAs as a primer for reverse transcription and replication is a hallmark of LTR-retroelements, which include endogenous retroviruses (ERVs) in mammals but also infectious retroviruses (Chapman et al., 1992; Schorn and Martienssen, 2018). In the mouse, ERVs are highly active causing mutations and are expressed during epigenetic reprogramming in stem cells of the developing embryo where also 3'-tRFs levels are high (Gagnier et al., 2019; Guo et al., 2024; Matsui et al., 2010; Rowe et al., 2010; Schorn et al., 2017). The two most active ERV families in mouse, IAP (*Intracisternal A Particle*) and ETn - MusD (*Early Transposon - Mus musculus particle type D*), are strongly inhibited by 3'-tRFs in retrotransposition assays (Schorn et al., 2017). Different LTR-retroelements have evolved to bind different primer tRNAs and are hence susceptible to 3'-tRFs from that specific tRNA sequence. If the PBS sequence is mutated, the transposon evades silencing, but may lose its ability to replicate. ETn/MusD and HIV-1 replicate using Lysine^{3}-TTT or Lys-TTT-3, one of seven Lysine-TTT isodecoder tRNAs that differ only in a few nucleotides.

3'-tRFs are processed from full-length, mature tRNAs including their posttranscriptional CCA-tail and come in two distinct sizes: 22 nucleotides long tRF3b repress coding-competent retroelements post-transcriptionally, while 17-19 nucleotides short tRF3a fragments interfere with reverse transcription (Schorn et al., 2017). 3'-tRFs have been found to bind PIWI and AGO proteins in many organisms, both during overexpression and in pulldowns of endogenous proteins (Couvillion et al., 2010; Hasler et al., 2016; Haussecker et al., 2010; Kumar et al., 2014; Li et al., 2012; Maute et al., 2013; Su et al., 2022; Yeung et al., 2009). Post-transcriptional gene silencing by tRF3b fragments has been demonstrated in reporter gene assays and for a number of genes with target sites in their 3'-UTR (Kuscu et al., 2018; Maute et al., 2013; Parikh et al., 2022; Su et al., 2022). The most widespread biological targets of 3'-tRFs are retroviral sequences that have complementarity at the PBS motif in their 5'-UTR (Kawaji et al., 2008; Li et al., 2012; Schorn et al., 2017; Yeung et al., 2009), but their target site rules are unknown. In contrast, target site rules

of miRNAs and piRNAs have been studied extensively (Bartel, 2009; Gainetdinov et al., 2023; McGeary et al., 2022; McGeary et al., 2019; Vainberg Slutskin et al., 2018).

Here, we provide a first systematic analysis of how mutations in the 5'-UTR target site of 3'-tRFs affect the expression of the highly active murine MusD retroelement. Silencing was effective despite mismatches to 3'-tRFs in the region defined as "seed" for miRNAs, but disrupted by mutations outside the seed region reminiscent of piRNAs. Distinct mechanisms regulate small RNA targeting and turnover depending on their sequence complementarity to their targets (Gainetdinov et al., 2021; Han and Mendell, 2023). We found 3'-tRFs are stabilized by 2'-O methylation like other small RNA classes with extensive target complementarity. In the absence of 2'-O methylation, 3'-tRFs accumulated adenosine and uridine tails mediated by TENT2 and TUT4, respectively. Terminal RNA modifications of 3'-tRFs and their target site rules suggest that 3'-tRFs are an ancient substrate of the RNAi machinery. We quantified 3'-tRFs and their precursor full-length tRNA molecules and found specific tRFs are highly enriched in a pattern conserved between mouse and human. The availability of full-length tRNA primer and inhibitory 3'-tRFs, their methylation status, as well as mutations at the PBS sequence are constrains that shape the evolutionary success of each ERV family. We found that HENMT1 methylates 3'-tRFs in germ cells but also has a function outside the mammalian germline in repressing LTR-retrotransposition, possibly extending its role to cancer and inflammatory diseases that overexpress TEs.

RESULTS

Selective enrichment of 3'-tRNA fragments (3'-tRFs) compared to full-length tRNA

To gain a comprehensive understanding of how tRNA levels relate to 3'-tRF expression in the cell, we isolated mature tRNA and 3'-tRF based on size from the same samples profiling different human and mouse stem cell lines (Figure 1A, B). We chose mouse embryonic P19 cells and mouse trophoblast stem cells (mTSC) (Tanaka et al., 1998) because of their high expression of ERVs and 3'-tRFs, respectively (Maksakova and Mager, 2005; Schorn et al., 2017). We also profiled human HeLa cells to observe potential trends across species and because we previously established regulation of the highly active murine ERV families ETn, MusD and IAP by 3'-tRFs in this heterologous host. We quantified full-length, mature tRNAs using hydrolysis-based tRNA sequencing and 3'-tRFs using standard ligation-based small RNA sequencing. Ligation-based small RNA sequencing readily detects 3'-tRFs that carry 5'-phosphate groups akin to miRNA, siRNA, piRNA and other endonuclease type III cleavage products. Hydrolysis-based sequencing is one of several methods to accurately quantify full-length tRNAs (Gogakos et al., 2017) despite their strong secondary structures and abundant RNA modifications that hamper reverse transcription. After optimizing hydrolysis conditions (Figure S1A), hydrolysis products that have 5'-hydroxyl groups are phosphorylated to allow for ligation and cloning. Both, tRNA hydrolysis products and 3'-tRFs, do not readily align to the genome due to their post-transcriptional CCA-tails and require tailored sequence analysis.

Analysis of tRNA, tRF3a, and tRF3b composition revealed that distinct amino acid isotypes were enriched for each class (Figure 1C, S1B). In general, tRNA composition exhibited a relatively

uniform distribution across isotypes with the most highly expressed isotypes representing ~10-15% of tRNA sequences (Figure 1C, S1B). In contrast, tRFs exhibited a much more skewed distribution, with the most abundant isotypes representing up to ~26% of 3'-tRFs. Importantly, the composition of 3'-tRFs differed from that of tRNAs and was consistent between replicates (Figure 1C, S1B). Moreover, tRF3a and tRF3b amounts were not related. For example, the most abundant isotype for tRF3a fragments was Alanine across all mouse and human cell lines sampled, while Lysine and Asparagine were the most abundant isotypes for tRF3b. Isotype tRNAs further split up into distinct isoacceptor tRNAs that encode the same amino acid but differ in their anticodon. Mapping reads to isoacceptor sequences resulted in distinct read counts demonstrating that the 3'-terminal sequence of each isoacceptor is unique and sufficient for unambiguous mapping of 3'-tRFs. Isoacceptor ratios differ significantly between tRNAs and tRFs (Figure 1E, F). Lysine has two isoacceptor families, Lys-CTT and Lys-TTT, the latter being the primer tRNA for several retroviruses including HIV-1 and ETn/MusD. While Lys-TTT is slightly underrepresented in the tested cell lines compared to Lysine-CTT, it starkly dominates the pool of tRF3a and tRF3b (Figure 1E).

Remarkably, tRFs of specific isoacceptors were highly enriched in a pattern conserved between mouse and human (Figure 1D, F, S1C, S1D). tRF3b fragments of eleven isoacceptors were significantly enriched in all three cell lines and enrichment of eight isoacceptors was shared for tRF3a (Figure 1F). While Alanine (all isoacceptors) and Lysine-TTT enrichment was observed for both tRF3b and tRF3a, other isoacceptor sequences were enriched specifically for either tRF3b or tRF3a. It should be noted that for the majority of isoacceptor sequences, 3'-tRF levels were roughly proportional to the amount of their parental, full-length tRNA. In addition to accumulation of 3'-tRFs from specific tRNAs, some tRFs were expressed less than expected. 3'-tRFs significantly underrepresented in all three cell lines for both, tRF3a and tRF3b, were SeC-TCA, Met-CAT, Ile-TAT, His-GTG (Figure 1F). Within each isoacceptor group, different isodecoder tRNAs exist that differ only in a few nucleotides but share the same anticodon. They too exhibit differential enrichment indicating that 3'-tRF amounts are highly regulated (Figure S1E). Lys-TTT-3 or Lys^3, the exact isodecoder that primes reverse transcription of HIV-1 and ETn/MusD, was significantly enriched across all cell types for both tRF3a and tRF3b (Figure S1E, F). Given that tRFs from certain tRNA isodecoders that differ only by a few nucleotides are much more abundant than others, this is unlikely due to selective endonucleolytic cleavage of tRFs from tRNAs, but rather suggests selective stability and turnover mechanisms.

3’-tRFs are 2'-O methylated and depend on the small RNA methyltransferase HENMT1

We set out to examine post-transcriptional RNA modifications of 3’-tRFs that might account for selective turnover and stability of 3'-tRFs and license them for *bona fide* small RNA silencing. 3'-tRFs have a strong 5'-nucleotide preference for uridine with tRF3b fragments ending in pseudo-uridine of the TψC-arm of tRNAs. Interestingly, pseudo-uridine in small RNAs of the plant germline affects transport and inheritance and also marks select miRNAs and piRNAs in animals (Herridge et al., 2025). Small RNAs with extensive sequence complementarity to their targets are stabilized by 2'-O methylation at their 3’-terminus: these include siRNAs in yeast, plants and invertebrates, as well as piRNAs in animals, but not miRNAs in animals that have been adapted for gene regulation and function by partial complementarity. 3'-tRFs have extensive complementarity to

their ERV targets owing to the conserved tRNA PBS motif. We analyzed mouse testis small RNA that was oxidized to enrich for 2'-O methylated RNA (Yang et al., 2019b) and found strong enrichment of 3'-tRFs (Figure 2A, B, C) akin to piRNAs that are known to be 2'-O methylated. Of 34 tRF3a and 49 tRF3b isoacceptors expressed above background (>5 BaseMean counts), 13 and 29, respectively, were significantly enriched (log2FC > 1 and padj. < 0.01). For this analysis, we included all sequences annotated as tRNA-derived in the UCSC repeat mask. These are named after the codon, i.e., Lys-AAA loci represent Lys-TTT tRNAs. Both, tRF3a and tRF3b Lysine-TTT fragments, were more than 8-fold enriched, indicating these sequences were robustly methylated. Notably, there is some heterogeneity among piRNA sequences as has been reported by others (Gainetdinov et al., 2021). Metazoan miRNAs are generally not 2'-O methylated and were depleted in oxidized samples as expected (Figure 2A). Similar to piRNAs, select 3'-tRF isotypes were heavily affected by oxidation. Ser, Thr, Pro, and Ala tRF3b, as well as Gly and Ser tRF3a fragments, were significantly depleted, thus providing evidence that their 3'-ends were not modified by methylation.

2'-O methylation of small RNAs is mediated by HENMT1 and is known to support piRNA stability (Horwich et al., 2007; Kamminga et al., 2010; Kirino and Mourelatos, 2007; Kurth and Mochizuki, 2009; Lim et al., 2015; Modepalli et al., 2018; Ohara et al., 2007; Pastore et al., 2021; Saito et al., 2007). Indeed, tRF3a and tRF3b were strongly depleted in Henmt1$^{em1Pdz/em1Pdz}$ loss-of-function mouse germline tissues (Figure 2D, E, F) (Gainetdinov et al., 2021). Small RNA length distribution profiles show loss of piRNA, but also 18 nt tRF3a and 22 nt tRF3b fragments in male Henmt1$^{em1Pdz/em1Pdz}$ germ cells (Figure 2D, S2A, S2B). 3'-tRFs were significantly destabilized in primary spermatocytes (spI), secondary spermatocytes (spII), as well as round spermatids (rs) of Henmt1$^{em1Pdz/em1Pdz}$ mice (Figure 2E, F, S2C). Trimming of piRNA precursors during biogenesis is mediated by the 3'-to-5' exonuclease PNLDC1. While piRNAs are susceptible to degradation in the absence of either HENMT1 or PNLDC1 (Figure S2D) (Gainetdinov et al., 2021), 3'-tRFs levels were not significantly affected by PNLDC1. This is consistent with the 3' ends of 3'-tRFs uniformly consisting of their post-transcriptional CCA-tail.

In the absence of HENMT1, 3'-tRFs are tailed by terminal nucleotidyltransferases

In the absence of 2'-O methylation, piRNAs accumulate non-templated nucleotide tails at their 3'-ends (Kamminga et al., 2010; Lim et al., 2015; Svendsen et al., 2019). 3'-tails that accompany degradation of miRNAs are mediated by the terminal nucleotidyltransferases TENT2, TUT4 and TUT7 (Han and Mendell, 2023). We analyzed tail length of miRNA, piRNA and 3'-tRFs in male germ cells of *Henmt1*$^{em1Pdz/em1Pdz}$ and *Pnldc1*$^{em1Pdz/em1Pdz}$ mice (Figure 3A, S3D) (Gainetdinov et al., 2021). As expected, neither loss of function had any effect on miRNAs which are not methylated in animals and are not trimmed by PNLDC1 (Figure 3A). In the absence of the trimmer enzyme, piRNAs accumulate a high percentage of 1-5 nt long tails. Tails did not accumulate on piRNAs in *Henmt1*$^{em1Pdz/em1Pdz}$, potentially because trimmer is still there to remove them or because only a fraction of piRNAs are 2'-O methylated and therefore affected. In contrast, 3'-tRFs accumulate tails of 2-3 nt length in either mutant and for both tRF3a and tRF3b fragments. Mono-, di-, and tri-nucleotide additions to 3'-tRFs increased up to three-fold in *Henmt1*$^{em1Pdz/em1Pdz}$ germ cells. When looking at the nucleotide composition of tails, miRNA and piRNA tails are dominated by single uridines, while 3'-tRFs carry longer U-tails (Figure 3B). Interestingly, 3'-tRFs in

spermatogonia exhibited adenine tailing in addition to untemplated uridine, a pattern also observed for miRNA. Spermatogonia are also the only tissue sampled in which 3'-tRFs were not depleted upon Henmt1 loss (Figure S2B, S2C), again suggesting 3'-tRFs in this tissue are more miRNA-like.

To determine which enzymes mediate tailing of 3'-tRFs in the absence 2'-O methylation, we looked for somatic tissues or cell lines that do not express HENMT1. While HEN1 homologs are expressed in both, germline and soma of plants and invertebrates, HENMT1 in animals is known for its role in the germline only. 3'-tRFs are expressed in early development including the germline (our unpublished data), but also in somatic tissues and many cancer cell lines. Interestingly, publicly available GTex RNA-seq data revealed a range of HENMT1 expression across tissues (Figure S3A). On average, cell lines derived from the cervix, testis, and adrenal gland exhibited the highest median expression, whereas those originating from the nervous system, liver, and kidney displayed the lowest expression. We corroborated these findings by Western blot, showing high expression of HENMT1 in HeLa cells (derived from the cervix), but no detectable expression in HEK293T cells (derived from the kidney) (Figure S3B). Thus, we analyzed small RNA data from AGO2-pulldown samples (Yang et al., 2022) of single, double, and triple knockouts of TUT4, TUT7 and TENT2 in HEK293T cells for tailing of 3'-tRF. Adenylation and uridylation were the predominant non-templated nucleotides added to 3'-tRFs in HEK293T cells (Figure 3C, D). Notably, tRF3a fragments in wildtype cells exhibited comparable amounts of adenylation and uridylation, whereas both miRNAs and tRF3b showed a two-fold bias towards uridylation. Strikingly, a cumulative of more than 40% of tRF3a reads had non-templated tails at their 3'-end, while only ~10-14% of tRF3b and miRNAs showed evidence of A- or U-tailing (Figure 3C, D). As previously reported, TENT2 mediates miRNA adenylation and its knockout (KO) resulted in a reduction of A-tails (Figure 3C). The impact on 3'-tRFs was even more pronounced, with a near complete abolition of A-tailing. Uridylation substantially decreased upon the depletion of TUT4 but did not decrease upon loss of TUT7 (Figure 3D). Accordingly, TUT4/7 double-knockouts (DKO) essentially show TUT4 uridylation level. Uridylation of tRF3a fragments was further diminished in the triple knockout (TKO) suggesting that TENT2 can uridylate them in the absence of other enzymes, underscoring its promiscuity for nucleotide incorporation in non-templated tails that has been reported before. Tail length and composition were the same when comparing AGO1- or AGO2-bound small RNAs to total, small RNA (Figure S3E). While the majority of miRNAs and tRF3b fragments were not tailed, tRF3a fragments were on average mono-tailed with up to three nucleotides (Figure 3E, F) and loss of all three transferases resulted in a substantial reduction of tRF3a tails relative to wildtype (Figure 3F). 3'-tRFs from certain tRNA isoacceptors such as Lys-TTT exhibited longer tails than others (Figure S3F, S3G). In summary, we observed substantial tailing of tRF3a fragments in the absence of HENMT1. Adenine tails of 3'-tRF depend on TENT2 with particularly long A-tails for tRF3a fragments, while uridine tails are short (1 nt) and depend on TUT4.

HENMT1 has a function outside the animal germline and enforces silencing of LTR-retrotransposition

To determine whether 3'-tRF methylation by HENMT1 has a function in transposon silencing, we used retrotransposition assays. The coding-competent MusD6 co-mobilizes the non-coding

ETnIIbeta retrotransposon by recognition of their shared, flanking LTR sequences (Ribet et al., 2004). The reporter gene becomes active only after splicing, reverse transcription and integration into the genome (Figure 4A). Retrotransposition assays are performed in a heterologous host to avoid confounding background of thousands of endogenous transposon copies in the original host species. We and others previously established HeLa cells as a heterologous host that is permissive for ETn/MusD retrotransposition and subject to regulation by 3'-tRFs (Ribet et al., 2004; Schorn et al., 2017). Human HeLa cells were transfected with the murine ERV reporter plasmids (Figure 4A, B), and selected for antibiotic resistance encoded by the reporter gene. Retrotransposition was quantified as the number of resistant cell colonies.

Indeed, knockdown of HENMT1 resulted in increased retrotransposition (Figure S4A) dependent on the PBS of MusD, the target site for Lysine 3'-tRFs, suggesting HENMT1 controls transposition by methylation of 3'-tRFs. While deletion of the ETn PBS sequence would abrogate tRNA priming for reverse transcription and hence transposition of the ETn-reporter, the MusD PBS sequence can be modified to "escape" regulation by 3'-tRFs with MusD gene products still retrotransposing the ETn-reporter *in trans* (Schorn et al., 2017). We tested whether increased transposition is due to increased expression and post-transcriptional regulation of MusD dependent on the PBS in the 5'-UTR using luciferase reporter constructs (Figure S4B). Only the reporter with an intact 18 nt PBS sequence was affected by HENMT1 knockdown. In order to test functional rescue of HENMT1 activity, we generated stable HENMT1 knockout clones (Figure S4C, S4D. We tested eight CRISPR guide RNAs targeting HENMT1 protein domains for their knockout efficiency by Western Blot (Figure S4C) and generated clonal cell lines (Figure S4D). Henmt1-deficient cell lines show reduced Lys3-tRF3b levels (Figure S4E). In order to specifically test the methyltransferase activity of HENMT1, we transiently expressed wildtype and catalytically inactive Henmt1- E132A (Figure 4C) (Peng et al., 2018) in Henmt1-deficient cells. The E132A mutation abolishes methyltransferase activity and specifically relieved silencing of transposition dependent on the 3'-tRF target site of MusD (Figure 4D). Lys^3-tRF3b levels were also rescued in cells with wildtype HENMT1 expression (Figure 4E). We confirmed these results for different amounts and ratios of the coding-competent MusD and the non-coding ETn-reporter (Figure S4F). In all conditions tested, ETn retrotransposition in Henmt1-deficient cells was only affected when driven by wildtype MusD with the intact PBS target site for 3'-tRFs. In contrast, MusD with mutation in the PBS (MusD-PBS^{mut}) escapes 3'-tRF regulation and the increased MusD protein amounts drive increased retrotransposition of the ETn reporter (Schorn et al., 2017). ETn retrotransposition mediated by MusD-PBS^{mut} was unaffected by the HENMT1 functional rescue (Figure 4D, S4F).

3'-tRF target sites in the mouse genome and target site rules for active endogenous retroviruses

Due to the perfect sequence complementarity to endogenous retroviral sequences, 3'-tRFs have many thousands of potential target sites in mammalian genomes. Under stringent criteria allowing no more than three mismatches, tRF3b fragments have ~9,000 sites of sequence complementarity in the mouse genome (Figure 5A). 5,047 of those are antisense to a repeat annotation (rmsk), 714 are potential target sites in annotated Gencode transcripts, 618 sites are within a Gencode transcript overlapping a repeat annotation, and 2,590 map to non-coding sequences on either strand. $Lysine^3$ 3'-tRFs primarily have target sites in TE-derived, repetitive

sequences (Figure 5B). Of those 221 transposon-derived target sites, a total of 215 are sequences of the ETn superfamily, which includes MusD6 (ETnERV2-int) and ETnIIbeta (MMETn-int) (Figure 5C). As expected, the large majority of transposon targets are ERVK elements by virtue of their Lysine (K) PBS.

How many mismatches in the PBS would disrupt post-transcriptional silencing by 3'-tRFs, but still allow for successful tRNA priming and proliferation of the retroelement? We previously analyzed the highly active murine ERV families IAP, MusD, and ETn for sequence conservation at their PBS (Cullen and Schorn, 2020) and noticed that many highly active elements do not have PBS sites that perfectly match their tRNA primer. This suggests successful elements evolve to avoid silencing at the PBS. We wanted to systematically test how complementarity to 3'-tRFs affects expression of ERVs. We collected all MusD full-length elements that fulfilled the following criteria: (i) ERVK:ETnERV-int entries (UCSC Golden Path rmsk annotation) larger than 6 kb, that (ii) are flanked by two LTRs of the ETn superfamily, and that (iii) have a PBS sequence immediately downstream of their 5'-LTR. Twenty-one of those had an intact Gag ORF as well as a PBS site among the top 4 most abundant PBS sequences (Figure 5J). Remarkably, we found that for all these full-length elements, sequence complementarity between the Lys^3 tRNA primer extends further downstream of the 18 nt PBS up to position 22. This suggests that this ERV family may actually use a 22 nt PBS for replication and certainly is able to base-pair with 22 nt long tRF3b fragments during post-transcriptional silencing. All elements have an A-to-C mismatch at the position that corresponds to the highly conserved 1-methyl-adenosine (m^1A) position in tRNAs (Figure 5J).

Which mismatches in the PBS would disrupt post-transcriptional silencing by 3'-tRFs? We employed a massively parallel reporter assay (MPRA) (Kinney and McCandlish, 2019) to determine which positions and nucleotides might be critical for 3'-tRF mediated post-transcriptional silencing. We used a reporter plasmid that is sensitive to silencing by 3'-tRFs and contains the 5'-UTR of MusD6 including its PBS upstream of a luciferase reporter gene (Schorn et al., 2017). We tested ~1,700 sequence variations at the Lys^3-TTT PBS motif. We included the PBS sequences from our analysis of the mouse genome, but also systematically introduced mutations in sliding windows of 3 nucleotides along the extended 22 nt PBS sequence. In addition, we used the miRNA target prediction software miRanda (Enright et al., 2003) to design scrambled sequences with poor hybridization scores for binding to Lys3-tRF3b. Cells were transfected with bulk-cloned, pooled reporter plasmid DNA, after which RNA was extracted to measure expression using a targeted RNA-seq protocol (Figure 5D). RNA expression was normalized to input DNA amount and quantified by NGS sequencing.

In analogy to nomenclature for base pairing between miRNA guide and target mRNA (McGeary et al., 2022), we defined a "seed" region (guide position g2-8), "central region" (g9-13) and "3'-supplemental region" (g14-22) along the 3'-tRF small RNA guide and its PBS target site (Figure 5J). In contrast to miRNAs, mismatches in the seed region did not necessarily relieve silencing (Figure 5E, F). Rather, mutations in the 3'-region de-repressed MusD-reporter expression. De-repression was defined as an increase compared to the "canonical" PBS sequence, which is perfectly complementary to its Lys^3-tRNA primer except for the conserved mismatch at the m^1A

position. RNA expression levels were highly reproducible between biological and technical replicates (n = 9) (Figure 5E, S5A, B, C). A few sequences were over- or under-represented in sequencing read depth, i.e., abundance (Figure S5B), among them the parental plasmid with the MusD6 wildtype 5'-UTR, but overall library skew is low across all replicates and conditions (Figure S5C). Library skew was quantified using a Lorenz curve which displays the evenness in the distribution of sequencing reads across library members, all replicates and conditions had a Gini index of less than 0.21 indicating a very even distribution of reads (Figure S5C). In order to correlate RNA to protein expression changes, we cloned a few select reporter plasmids out of the pool and quantified protein expression using the luciferase reporter gene. Of note, the relatively small, but highly reproducible fold changes of RNA expression quantified by the MPRA translated into ~2-fold changes of protein expression in luciferase assays (Figure S5D). RNA and protein expression for a PBS sequence with zero mismatches to Lys^3-tRF3b had significantly lower expression than the wildtype MusD6 PBS with three mismatches suggesting that active retroelement copies have evolved to escape silencing. In other words, genomic coding-competent ERV copies with mismatches in their PBS seem to be at an advantage for expression.

To characterize the effects of PBS mutations in more detail, we first grouped sequence variants by the number of mutations per region relative to the canonical reference PBS (Figure 5G). Mutations in the 3'- and central regions strongly increased expression. Increasing mismatches in the central region means a greater central bulge, i.e., unpaired nucleotides between small RNA and target, and further increased expression. Mismatches within the seed region had variable effects on expression. Single mismatches did not significantly change de-repression. Double and triple mutations produced highly variable effects on de-repression but increased de-repression on average, suggesting repression or de-repression depends on nucleotide identity and position. In all regions, expression increased with the number of mismatches between PBS and Lys^3-tRF3b (Figure 5G, S5E).

Position and nucleotide identity can be scored for their impact on expression using algorithms specifically developed for MPRA. We used the MAVE-NN algorithm to create a genotype to phenotype map (Tareen et al., 2022) that scores additive contributions from each possible base at each position to a latent phenotype (DF) that is nonlinearly related to measured expression values (Figure 5H). Changes in F result from mutations disrupting the canonical PBS sequence. Strikingly, nucleotide changes at certain positions had a stark effect on expression. Changes at the target positions t7-9 and t15-22 generally disrupted silencing with increased expression indicated in red. Those positions coincide with highly conserved positions in 3'-tRFs such as the post-transcriptional CCA-tail. Three nucleotide sliding windows along the PBS sequence show de-repression in several areas that are often mutated in genomic MusD copies (Figure 5I). Mutations in the 5'-end of the PBS, close to the 3'-end and CCA-tail of the tRNA primer, will disrupt tRNA priming and replication. Therefore, while advantageous for expression, these elements will not be "successful" and not increase copy number in the genome. However, positions that do not restrict mobility of the retroelement and still allow reverse transcription, can accumulate mutations and be of advantage for high expression. Mutations that still allow tRNA priming are known from actively transposing, high copy number MusD elements (asterisks, Figure 5J) (Ribet et al., 2004) and can potentially be inferred (blue shaded) from mutagenesis studies on HIV-1 that uses the

same Lys^3-tRNA primer (Wang et al., 2018). PBS mutations that preserve tRNA priming but evade 3′-tRF silencing offer a selective advantage and these elements should accumulate to higher copy numbers in the genome. Indeed, many of the positions mutated in active full-length MusD copies in the mouse genome coincide with positions that conferred de-repression in the MPRA (Figure 5H, I, J). This suggests that, with many layers of regulation controlling transposition *in vivo*, systematic mutagenesis in an expression-only context as in this MPRA can effectively dissect regulatory constraints on ERVs.

DISCUSSION

Our data demonstrates a role for HENMT1 in "licensing" 3'-tRFs for transposon control. We found that 3'-tRFs are marked and stabilized by 2'-O methylation (Figure 2) much like other small RNA classes that exhibit extensive sequence complementarity to transposons and viruses, and that serve host defense. The highly active murine ERV MusD is specifically restricted by HENMT1's methyltransferase activity in retrotransposition assays (Figure 4). Our data indicate that transposon control by 3'-tRFs shares key characteristics with other small RNA silencing pathways. 3'-tRFs are processed from their precursor molecules, full-length tRNAs, in a highly regulated fashion (Figure 1). They share sequence and RNA modifications typical of *bona fide* regulatory small RNAs. This includes their 5'-uridine or pseudo-uridine as well as 2'-O methylation at their 3'-ends. Their transposon targets show signatures of selective pressure to escape 3'-tRF mediated silencing (Figure 5). In the absence of 2'-O methylation, 3'-tRFs become substrates of terminal nucleotidyltransferases (Figure 3) and accumulate A- and U-tails akin to piRNAs and miRNAs (Gainetdinov et al., 2020; Kamminga et al., 2010; Lim et al., 2015; Yang et al., 2022).

3'-tRFs showed distinct patterns of enrichment when compared to the composition of parental, full-length tRNAs in the cell (Figure 1), and are clearly not by-products of tRNA decay. In mouse and human, tRNA transcript levels from specific loci vary between cell types whereas tRNA anticodon pools do not, supporting stable translational outcomes (Gao et al., 2024; Schmitt et al., 2014). Consistent with this, we saw largely similar tRNA expression between human and mouse cell lines when grouped by anticodon (Figure S1C). 3'-tRF production can be manipulated by overexpression of single tRNAs (Kuscu et al., 2018), and the abundance of many 3'-tRFs is roughly proportional to their tRNA level (Figure 1C). However, our data clearly shows strong enrichment of specific 3'-tRFs isoacceptor and isodecoder sequences in a pattern conserved between mouse and human (Figure 1D, F). Although tRF3a and tRF3b fragments of the Lys^3-TTT (Lys-TTT-3) isodecoder are significantly enriched compared to their full-length tRNA in all cell lines tested, their expression levels varied starkly between cell lines (Figure S1F). Processive reverse transcription *in vivo* also requires interactions with the tRNA beyond the PBS sequence, and hence constrains LTR-retroelements to use specific isodecoder tRNA sequences that their proteins evolved to bind to (Keeney et al., 1995; Le Grice, 2003; Liu et al., 2010). Lys^3-TTT tRNA is expressed at low levels in both mouse stem cell lines, and so may restrict retrotransposition in these cells, despite their high expression level of ETn/MusD (Maksakova and Mager, 2005; Schorn et al., 2017; Xu and Boeke, 1990). HeLa cells on the other hand express large amounts of this primer tRNA (Figure S1F) and evidently support reverse transcription of ETn/MusD. Small RNA and 3'-tRF regulation however are permissive to mismatches (Figure 5) and Lys^3-TTT primed retroviruses may well be regulated by 3'-tRFs of other isodecoder sequences. tRNA

versus 3'-tRF levels could determine which cell types are permissive to mobility versus expression of the elements. In fact, 3'-tRFs have the capacity to affect both, expression level and reverse transcription in these cell lines. The 3'-end of related isodecoder tRNAs and therefore 3'-tRFs are similar enough in sequence (Figure S1G, S1H) that one ERV family could be regulated by several isodecoder 3'-tRFs.

tRF3b fragments mediate post-transcriptional silencing (Haussecker et al., 2010; Kuscu et al., 2018; Maute et al., 2013; Schorn et al., 2017) and have a large number of predicted target sites in metazoan genomes due to the abundance of LTR-retroelement derived sequences (Figure 5A, B). PBS sequences of active mouse ERV families show signatures of 3'-tRF mediated regulation (Cullen and Schorn, 2020). Mutations in the PBS of mobile copies can be interpreted as selective pressure to escape 3'-tRF mediated silencing (Figure 5J). Our results from the MPRA clearly show that mutations confer an advantage for expression of MusD. Interestingly, there is not a single perfect match of Lys3-tRF3b in the entire mouse genome. The most frequently observed mismatch corresponds to the m^1A in tRF3b fragments, at position 58 of tRNAs. This modification is an A-form RNA helix disrupter (Zhou et al., 2016) and might therefore not matter for base-pairing with tRF3b fragments. The observed cytosine in the ETn/MusD PBS is most likely due to the common misincorporation of nucleotides during reverse transcription of m^1A at this position of the tRNA (Ryvkin et al., 2013). It is worth keeping in mind that most TEs in mammalian genomes are non-autonomous. For retroelements that have no "cis" preference to mobilize their own RNA, protein-coding "master-copies" that accumulate mutations at their PBS may lose the ability to prime reverse transcription of themselves but can achieve high expression by evading post-transcriptional silencing and retrotranspose a large number of non-coding, non-autonomous elements with perfect PBS sites. Interestingly, a large number of PBS sequences in inactive and immobile elements are very well conserved. Even though their PBS motif is no longer required for tRNA priming, it may serve co-opted regulation of these transcripts by 3'-tRFs. Many of these elements have essential functions in early development and participate in gene-regulatory networks in mouse and human (Faulkner et al., 2009; Franke et al., 2017; Guo et al., 2024; Modzelewski et al., 2021; Peaston et al., 2004; Rebollo et al., 2012).

Target rules for miRNAs are well established and rules for piRNAs are emerging. In fact, a number of MPRA designs have been used to measure miRNA-target interactions (McGeary et al., 2019; Vainberg Slutskin et al., 2018). Strong binding between guide (positions g7-15 and g8-16) and targets have been observed for PIWI complexes even in the absence of seed base pairing (Anzelon et al., 2021; Gainetdinov et al., 2023). This is thought to benefit the ability of PIWI proteins to silence transposons that mutate frequently. Our data on de-repression of the MusD transposon sequence much resemble piRNA target interactions. MusD repression is sensitive to mutations in the 3'-region, but less sensitive to mutations in the seed sequence that dominates miRNA-target interactions (Figure 5). Although the MPRA will include potential transcriptional effects on reporter plasmid DNA by e.g., zinc finger (ZNF) proteins, only a few of the positions with strong effects in the MAVE-NN analysis (Figure 5H) overlap the binding motif of human ZNF417/587, which has evolved as another layer of defense against $Lysine^3$-primed retroviruses (Turelli et al., 2020). The biggest effects on expression were observed for mutations in the region that base-pairs with the highly conserved CCA-end of 3'-tRFs. Few examples of LTR-

retroelements being able to switch between isodecoder tRNAs for priming at low frequency have been observed (Cullen and Schorn, 2020; Das et al., 1997; Fennessey et al., 2019; Markopoulos et al., 2016). Therefore, 3'-tRF target sites are certainly under different evolutionary constraints than miRNA target sites. Also, every round of replication bears a 50:50 chance for *Retroviridae* like MusD of reverting their PBS sequence to a cDNA copy of their primer tRNA (Berwin and Barklis, 1993; Cullen and Schorn, 2020; Hu and Hughes, 2012), further constraining escape from silencing.

3'-tRFs are subjected to A-tailing by TENT2 and U-tailing by TUT4 similar to miRNAs. TUT4 and TUT7 were not redundant (Figure 3C, D), but U-tails on 3'-tRFs were TUT4-dependent. In the absence of 2'-O methylation by HENMT1, extent and length of tailing of tRF3b fragments are similar to that of miRNAs (Figure 3A, C, D). Both small RNA classes act in post-transcriptional silencing. We observed long, TENT2-dependent A-tails for tRF3a fragments in HEK293T cells (Figure 3C, E, F). A-tailed Lys^3-tRF3a are perfectly complementary beyond the ETn/MusD target site, and although overall levels are low, it is conceivable that tailed 3'-tRFs regulate an additional target repertoire similar to reports for tailed miRNA (Yang et al., 2019a). Untailed tRF3a are rather similar in size to tinyRNAs (tyRNAs) that have been described to guide AGO3-mediated silencing (Sim et al., 2023). Similar to overall tRF enrichment patterns, tailing was highly specific to some isodecoders but not to others (Figure S3F, G).

We found that 3'-tRFs are 2'-O methylated and depend on HENMT1 for stability. This is reminiscent of small RNAs in other organisms which depend on 2'-O methylation for stability (Ji and Chen, 2012; Kamminga et al., 2010; Kurth and Mochizuki, 2009; Modepalli et al., 2018; Zhao et al., 2012) and target highly complementary sequences such as transposons and viruses. Phylogenetic analyses show that vertebrates have lost the antiviral AGO clade (Wynant et al., 2017). Long, double-stranded RNA (dsRNA) generally invokes an interferon response in vertebrates restricting the generation of small RNAs from dsRNA of repetitive elements. Few tissues such as oocytes in mice and rats produce endogenous siRNAs using a specific Dicer isoform that efficiently cleaves long dsRNA and circumvents RNA immunity pathways (Flemr et al., 2013; Tam et al., 2008; Watanabe et al., 2008; Yang et al., 2019b). Oocytes of other mammals express a fourth PIWI protein (PIWIL3) to produce 19 nt short piRNAs (Loubalova et al., 2021; Lv et al., 2023; Wang et al., 2023; Yang et al., 2019b). Neither of those oocyte small RNA classes are 2'-O methylated and neither are metazoan miRNAs that evolved to silence targets via partial complementarity. It makes sense that 3'-tRFs are 2'-O methylated, akin to other small RNAs that aim to silence RNA with near perfect pattern match. 3'-tRF might represent an ancient defense mechanism to detect mobile repetitive RNA in early-stage embryonic cell types that cannot be surveilled by the vertebrate immune system.

Our data suggests a functional role of HENMT1 outside the germline of animals. HENMT1 methyltransferase activity restricts ETn/MusD retrotransposition in somatic cells (Figure 4D). Both, HENMT1 and 3'-tRFs are expressed in early development, but also in somatic tissues and in many cancer cell lines. Depletion of HENMT1 results in growth defects and scores as strongly selective across cancer cell lines (Figure S3C). HENMT1 was the top dysregulated RNA-modifying enzyme in a cancer study comparing tumors to matched normal samples (Begik et al.,

2020). The small RNA methyltransferase is also a prognostic marker in cervical cancer (CESC) according to the TCGA Protein Atlas data (https://www.proteinatlas.org/ENSG00000162639-HENMT1/cancer). None of these observations can be explained by HENMT1's role in piRNA biogenesis in the germline. Rather, HENMT1's role in the soma might be to methylate 3'-tRFs and promote ERV silencing. Somatic ERV re-activation leads to sustained inflammation and is a common theme in aging, cancer and auto-immune diseases (Jansz and Faulkner, 2021; Liu et al., 2023; Volkman and Stetson, 2014).

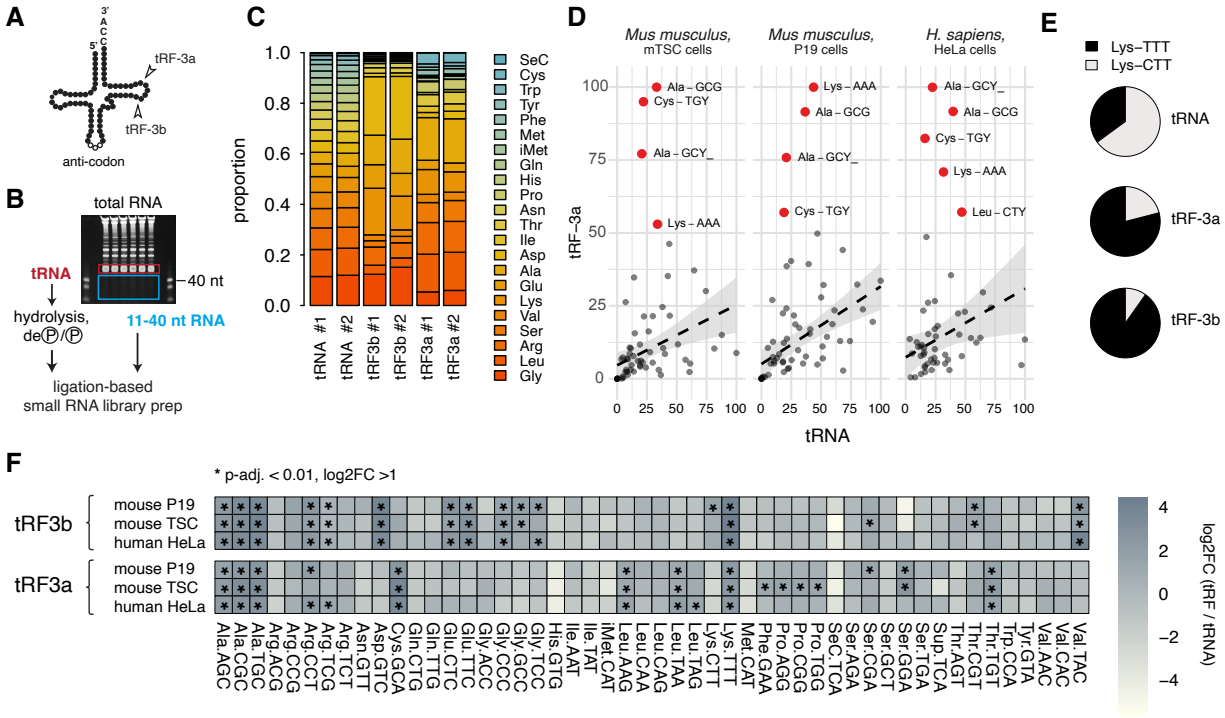

A
3'
A
C
5'
C
tRF-3a
tRF-3b
anti-codon
B
total RNA
tRNA
40 nt
hydrolysis,
deⓅ/Ⓟ
11-40 nt RNA
ligation-based
small RNA library prep
C
proportion
1.0
0.8
0.6
0.4
0.2
0.0
tRNA #1
tRNA #2
tRF3b #1
tRF3b #2
tRF3a #1
tRF3a #2
SeC
Cys
Trp
Tyr
Phe
Met
iMet
Gln
His
Pro
Asn
Thr
Ile
Asp
Ala
Glu
Lys
Val
Ser
Arg
Leu
Gly
D
Mus musculus, mTSC cells
Mus musculus, P19 cells
H. sapiens, HeLa cells
Ala – GCG
Cys – TGY
Ala – GCY_
Lys – AAA
Lys – AAA
Ala – GCG
Ala – GCY_
Cys – TGY
Ala – GCY_
Ala – GCG
Cys – TGY
Lys – AAA
Leu – CTY
tRF−3a
tRNA
E
Lys−TTT
Lys−CTT
tRNA
tRF-3a
tRF-3b
F
* p-adj. < 0.01, log2FC >1
tRF3b
mouse P19
mouse TSC
human HeLa
tRF3a
mouse P19
mouse TSC
human HeLa
Ala.AGC
Ala.CGC
Ala.TGC
Arg.ACG
Arg.CCG
Arg.CCT
Arg.TCG
Arg.TCT
Asn.GTT
Asp.GTC
Cys.GCA
Gln.CTG
Gln.TTG
Glu.CTC
Glu.TTC
Gly.ACC
Gly.CCC
Gly.GCC
Gly.TCC
His.GTG
Ile.AAT
Ile.TAT
iMet.CAT
Leu.AAG
Leu.CAA
Leu.CAG
Leu.TAA
Leu.TAG
Lys.CTT
Lys.TTT
Met.CAT
Phe.GAA
Pro.AGG
Pro.CGG
Pro.TGG
SeC.TCA
Ser.AGA
Ser.CGA
Ser.GCT
Ser.GGA
Ser.TGA
Sup.TCA
Thr.AGT
Thr.CGT
Thr.TGT
Trp.CCA
Tyr.GTA
Val.AAC
Val.CAC
Val.TAC
4
2
0
−2
−4
log2FC (tRF / tRNA)

Figure 1

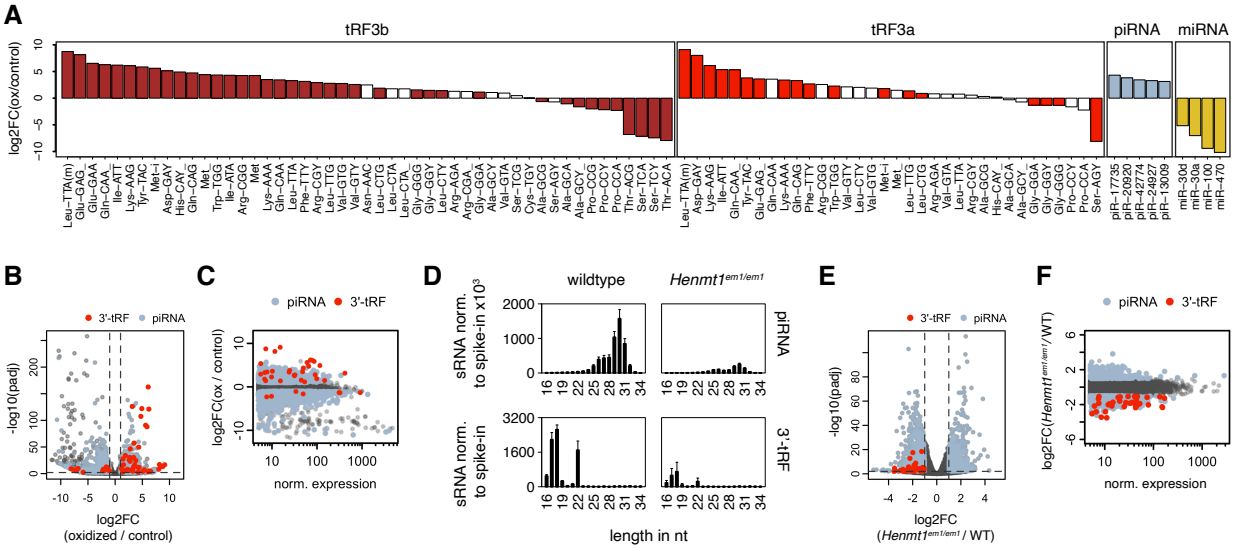

A
tRF3b
tRF3a
piRNA
miRNA
log2FC(ox/control)
B
3'-tRF
piRNA
-log10(padj)
log2FC
(oxidized / control)
C
piRNA
3'-tRF
log2FC(ox / control)
norm. expression
D
wildtype
Henmt1em1/em1
sRNA norm. to spike-in x10³
piRNA
3'-tRF
length in nt
E
3'-tRF
piRNA
-log10(padj)
log2FC
(Henmt1em1/em1 / WT)
F
piRNA
3'-tRF
log2FC(Henmt1em1/em1/WT)
norm. expression

Figure 2

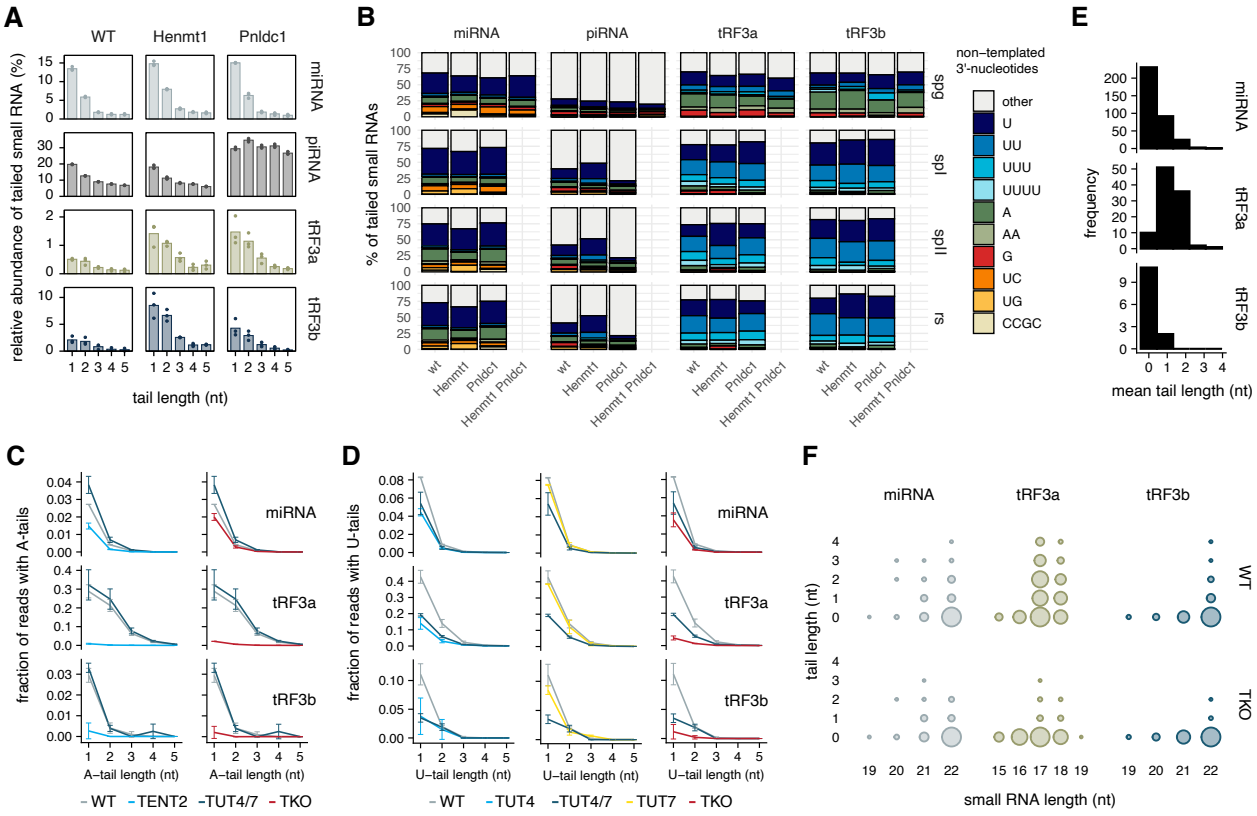

A
WT
Henmt1
Pnldc1
miRNA
piRNA
tRF3a
tRF3b
relative abundance of tailed small RNA (%)
tail length (nt)
B
miRNA
piRNA
tRF3a
tRF3b
% of tailed small RNAs
sgg
spl
spll
rs
wt
Henmt1
Pnldc1
Henmt1 Pnldc1
non−templated 3'-nucleotides
other
U
UU
UUU
UUUU
A
AA
G
UC
UG
CCGC
E
frequency
mean tail length (nt)
C
fraction of reads with A-tails
A−tail length (nt)
WT
TENT2
TUT4/7
TKO
D
fraction of reads with U-tails
U−tail length (nt)
WT
TUT4
TUT4/7
TUT7
TKO
F
tail length (nt)
small RNA length (nt)
WT
TKO

Figure 3

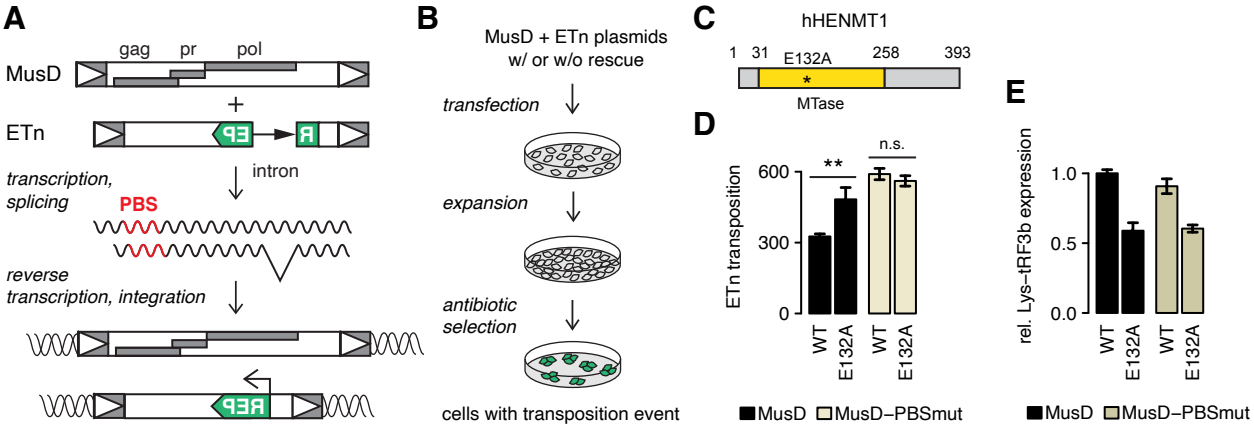

A
gag
pr
pol
MusD
+
ETn
EP
R
intron
transcription,
splicing
PBS
reverse
transcription, integration
REP
B
MusD + ETn plasmids
w/ or w/o rescue
transfection
expansion
antibiotic
selection
cells with transposition event
C
hHENMT1
1
31
E132A
258
393
MTase
D
ETn transposition
600
300
0
**
n.s.
WT
E132A
WT
E132A
MusD
MusD−PBSmut
E
rel. Lys−tRF3b expression
1.0
0.5
0.0
WT
E132A
WT
E132A
MusD
MusD−PBSmut

Figure 4

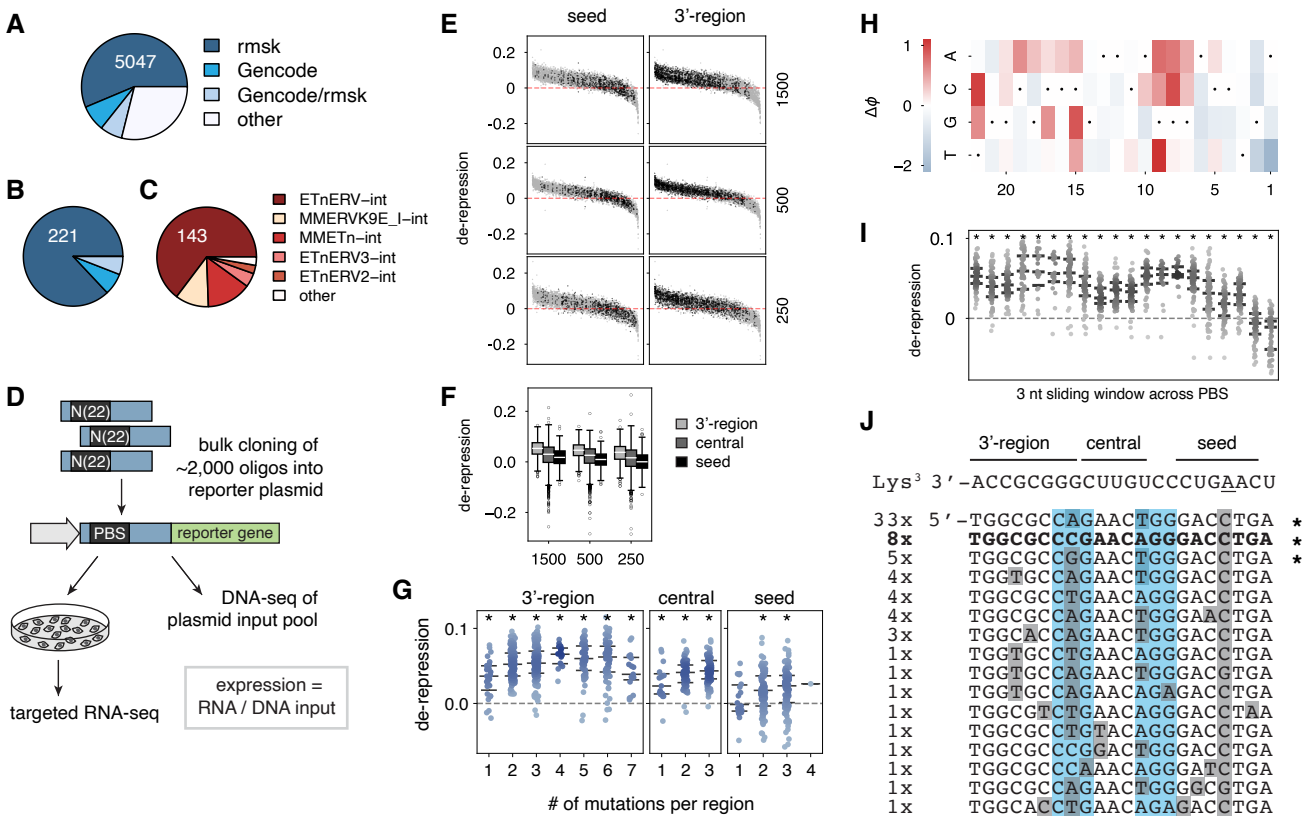

A
5047
rmsk
Gencode
Gencode/rmsk
other
B
221
C
143
ETnERV–int
MMERVK9E_I–int
MMETn–int
ETnERV3–int
ETnERV2–int
other
D
N(22)
N(22)
N(22)
bulk cloning of
~2,000 oligos into
reporter plasmid
PBS
reporter gene
DNA-seq of
plasmid input pool
targeted RNA-seq
expression =
RNA / DNA input
E
seed
3'-region
de-repression
1500
500
250
F
de-repression
3'-region
central
seed
1500 500 250
G
3'-region
central
seed
de-repression
# of mutations per region
H
Δϕ
A
C
G
T
20 15 10 5 1
I
de-repression
3 nt sliding window across PBS
J
3'-region
central
seed
Lys3 3'-ACCGCGGGCUUGUCCCUGAACU
33x 5'-TGGCGCCAGAACTGGGACCTGA
8x TGGCGCCCGAACAGGGACCTGA
5x TGGCGCCGGAACTGGGACCTGA
4x TGGTGCCAGAACTGGGACCTGA
4x TGGCGCCTGAACAGGGACCTGA
4x TGGCGCCAGAACTGGGAACTGA
3x TGGCACCAGAACTGGGACCTGA
1x TGGTGCCTGAACAGGGACCTGA
1x TGGTGCCAGAACTGGGACGTGA
1x TGGTGCCAGAACAGAGACCTGA
1x TGGCGTCTGAACAGGGACCTAA
1x TGGCGCCTGTACAGGGACCTGA
1x TGGCGCCCGGACTGGGACCTGA
1x TGGCGCCCAAACAGGGATCTGA
1x TGGCGCCAGAACTGGGGCGTGA
1x TGGCACCTGAACAGAGACCTGA

Figure 5

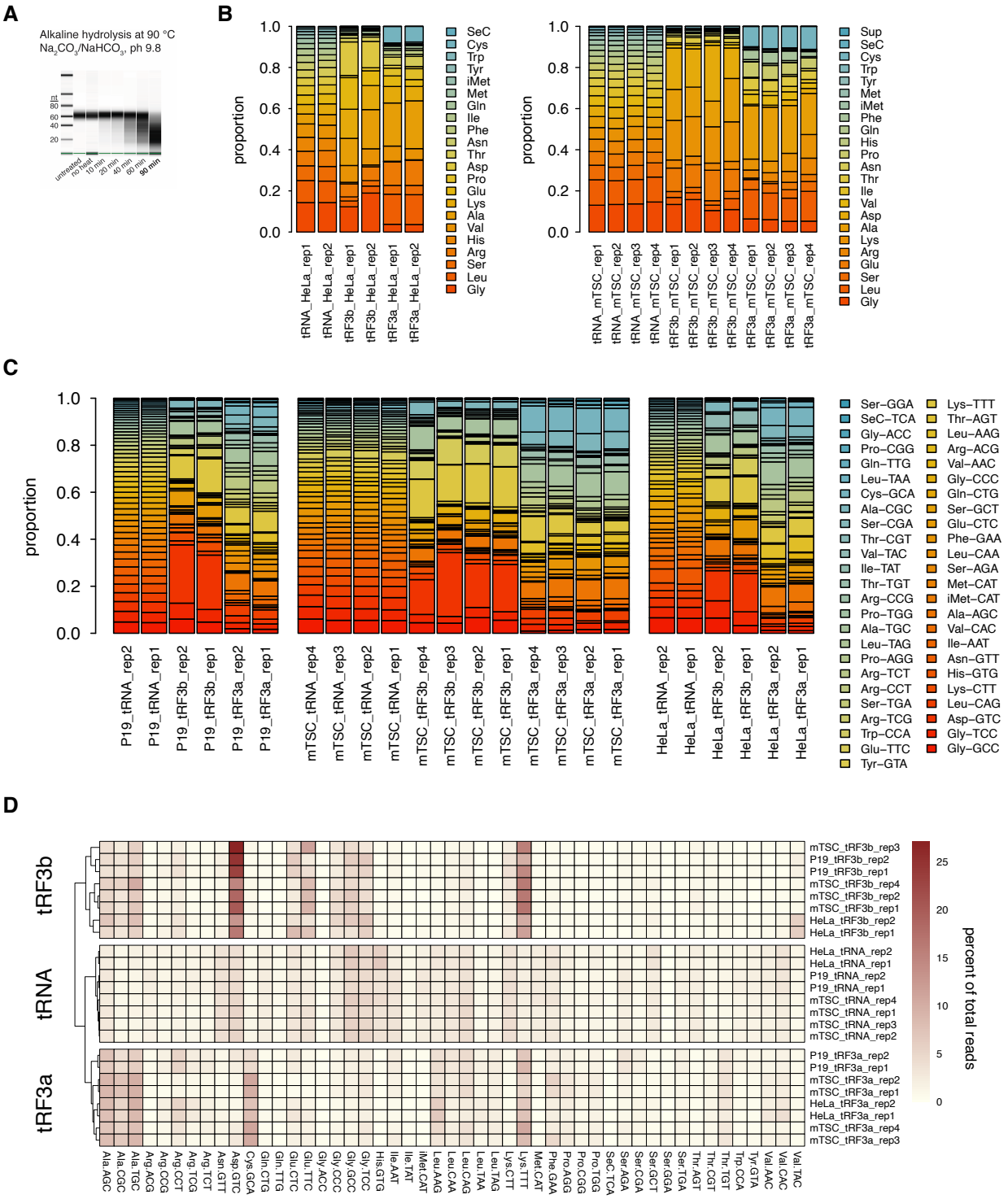


Figure S1 - page 1

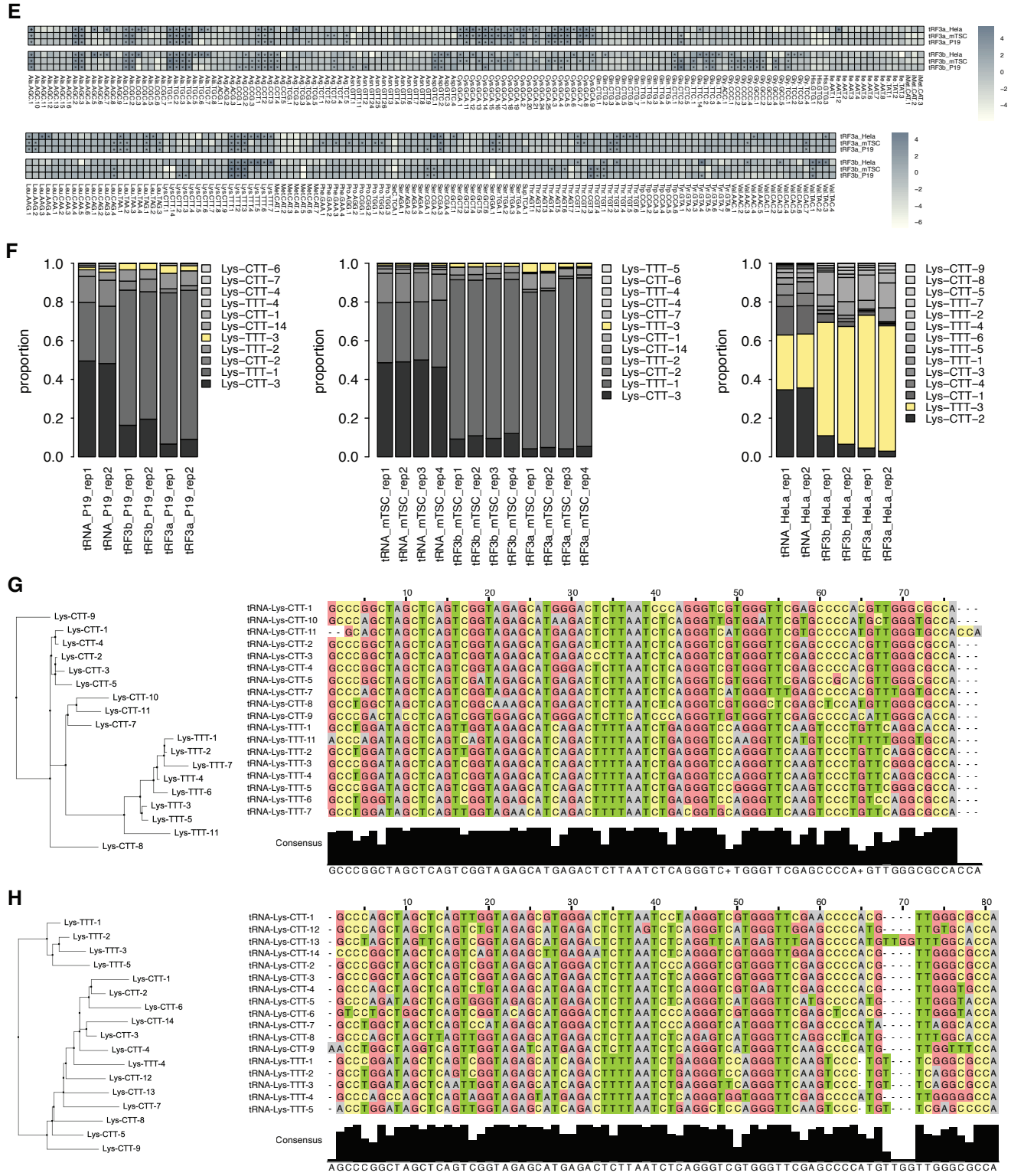


Figure S1, page 2

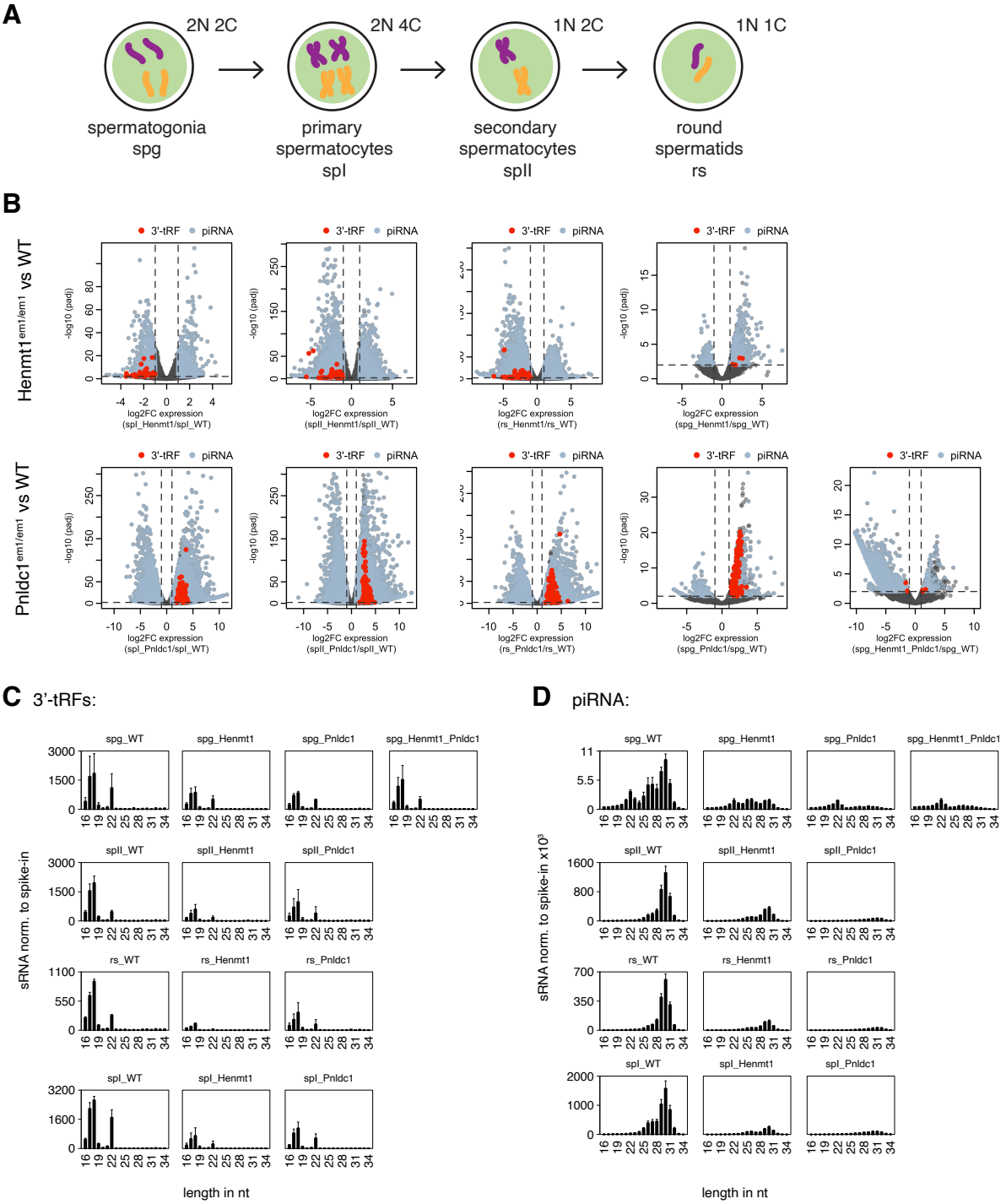

A
2N 2C
2N 4C
1N 2C
1N 1C
spermatogonia
spg
primary
spermatocytes
spI
secondary
spermatocytes
spII
round
spermatids
rs
B
Henmt1em1/em1 vs WT
Pnldc1em1/em1 vs WT
3'-tRF
piRNA
-log10 (padj)
log2FC expression
(spI_Henmt1/spI_WT)
(spII_Henmt1/spII_WT)
(rs_Henmt1/rs_WT)
(spg_Henmt1/spg_WT)
(spI_Pnldc1/spI_WT)
(spII_Pnldc1/spII_WT)
(rs_Pnldc1/rs_WT)
(spg_Pnldc1/spg_WT)
(spg_Henmt1_Pnldc1/spg_WT)
C 3'-tRFs:
D piRNA:
spg_WT
spg_Henmt1
spg_Pnldc1
spg_Henmt1_Pnldc1
spII_WT
spII_Henmt1
spII_Pnldc1
rs_WT
rs_Henmt1
rs_Pnldc1
spI_WT
spI_Henmt1
spI_Pnldc1
sRNA norm. to spike-in
sRNA norm. to spike-in x10³
length in nt

Figure S2

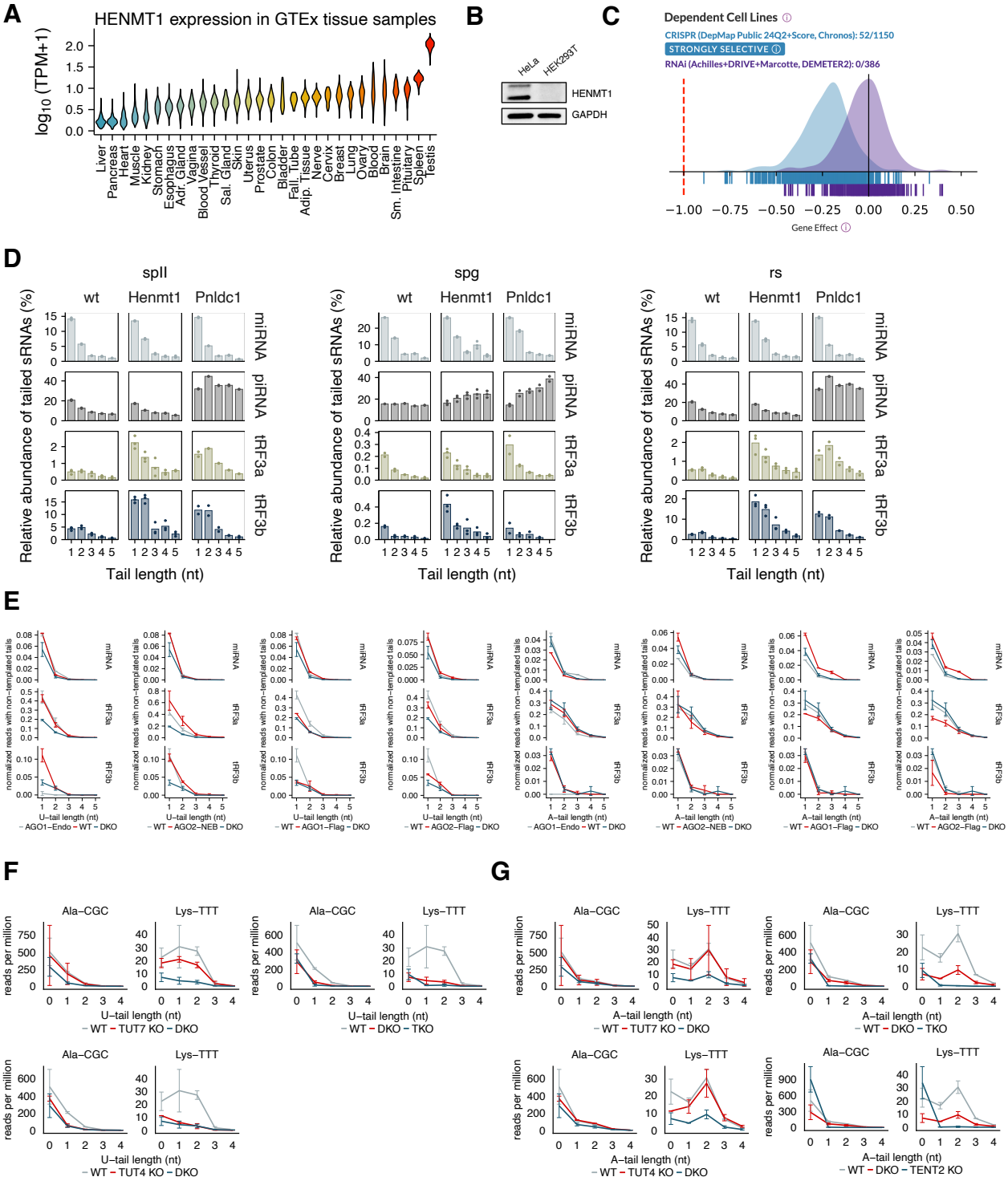
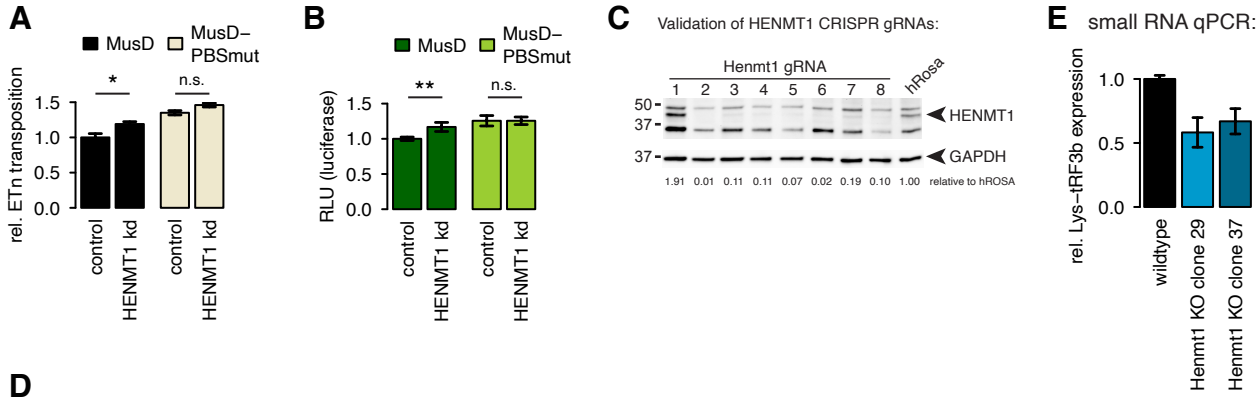

A
HENMT1 expression in GTEx tissue samples
log10 (TPM+1)
Liver
Pancreas
Heart
Muscle
Kidney
Stomach
Esophagus
Adr. Gland
Vagina
Blood Vessel
Thyroid
Sal. Gland
Skin
Uterus
Prostate
Colon
Bladder
Fall. Tube
Adip. Tissue
Nerve
Cervix
Breast
Lung
Ovary
Blood
Brain
Sm. Intestine
Pituitary
Spleen
Testis
B
HeLa
HEK293T
HENMT1
GAPDH
C
Dependent Cell Lines
CRISPR (DepMap Public 24Q2+Score, Chronos): 52/1150
STRONGLY SELECTIVE
RNAi (Achilles+DRIVE+Marcotte, DEMETER2): 0/386
Gene Effect
D
spII
spg
rs
wt
Henmt1
Pnldc1
miRNA
piRNA
tRF3a
tRF3b
Relative abundance of tailed sRNAs (%)
Tail length (nt)
E
normalized reads with non-templated tails
U-tail length (nt)
A-tail length (nt)
AGO1-Endo
WT
DKO
AGO2-NEB
AGO1-Flag
AGO2-Flag
F
Ala-CGC
Lys-TTT
reads per million
TUT7 KO
TKO
TUT4 KO
G
TENT2 KO

Figure S3



D

```
Clone #37
wildtype  (56) Cys Gly Asp Thr Ser Leu Leu Arg Leu Leu Lys Val Asn Pro
allele 1  (56) Cys Gly Asp Thr Ser Leu Ile Tyr Asp Cys  *
allele 2  (56) Cys Gly Asp Thr Ser Tyr Asp Cys  *
allele 3  (56) Cys Gly Asp Ile Thr Thr Ala Lys Ser Gln Ser Met His  *

Clone #29
wildtype   (50) Lys Val Ala Asp Leu Gly Cys Gly Asp Thr Ser Leu Leu
allele 1   (50) Lys Val Gly Ile Ser Leu Ser Lys His Leu Ile Ile Lys Thr Ser Arg Leu Val Ile *
allele 2   (50) Lys Leu Ile Ile Leu Leu Leu Ser *
allele 3   (50) Lys Gly Phe Val Ser Ser Phe Pro Gly Gly Phe Ser Glu Thr *
```

F retrotransposition in the presence of HENMT1 wildtype (WT) or catalytic mutant HENMT1 (E132A):

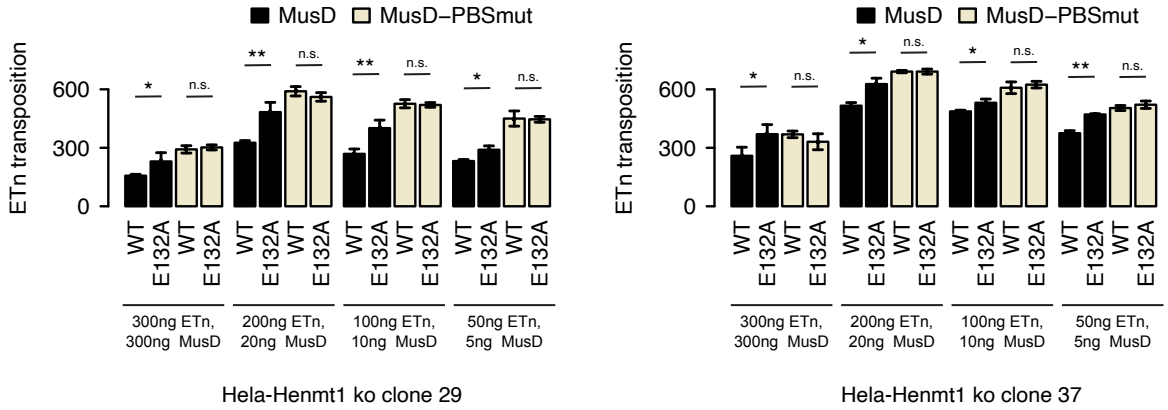


Figure S4

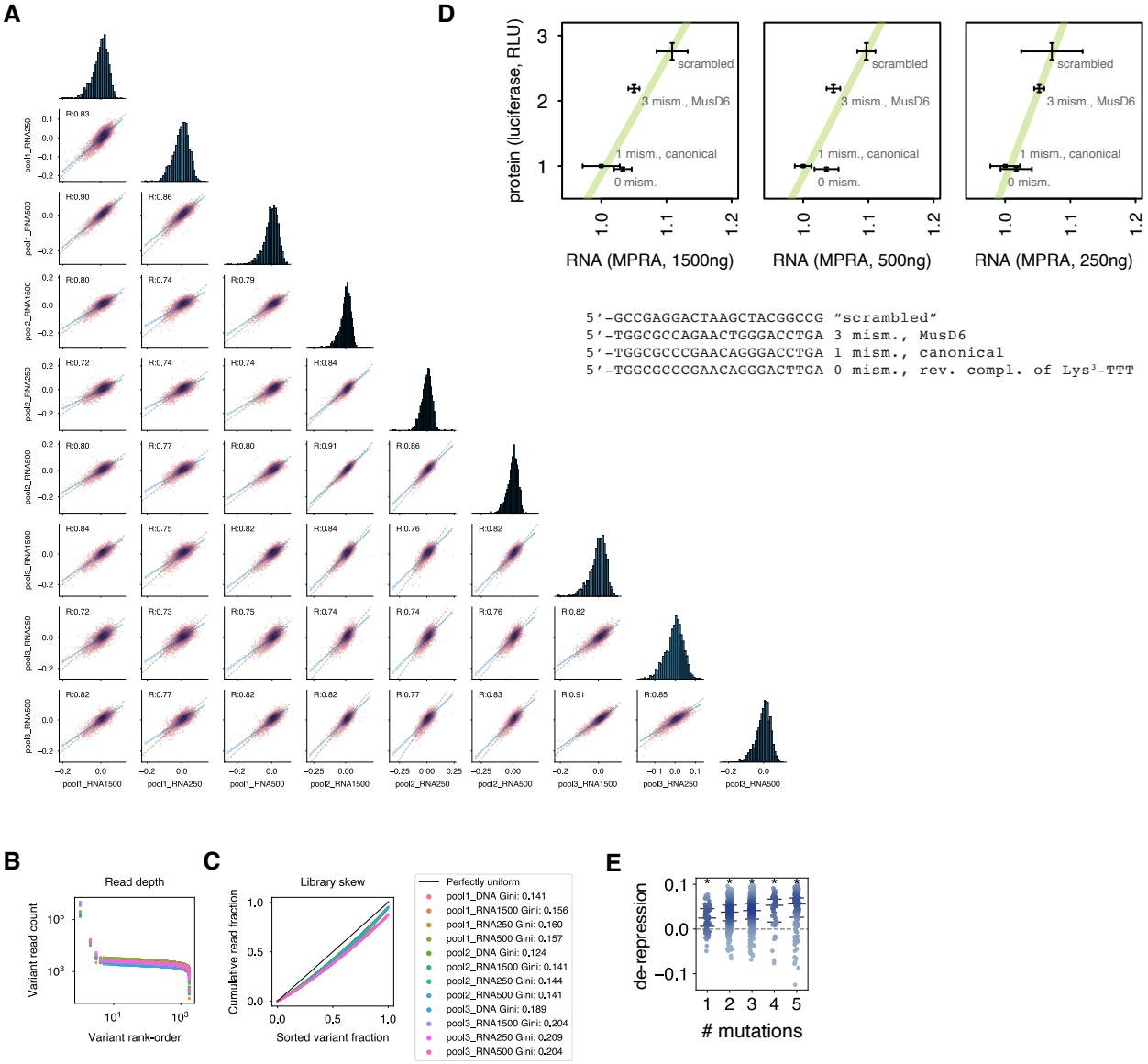

A
D
protein (luciferase, RLU)
scrambled
3 mism., MusD6
1 mism., canonical
0 mism.
RNA (MPRA, 1500ng)
RNA (MPRA, 500ng)
RNA (MPRA, 250ng)
5'-GCCGAGGACTAAGCTACGGCCG "scrambled"
5'-TGGCGCCAGAACTGGGACCTGA 3 mism., MusD6
5'-TGGCGCCCGAACAGGGACCTGA 1 mism., canonical
5'-TGGCGCCCGAACAGGGACTTGA 0 mism., rev. compl. of Lys3-TTT
pool1_RNA1500
pool1_RNA250
pool1_RNA500
pool2_RNA1500
pool2_RNA250
pool2_RNA500
pool3_RNA1500
pool3_RNA250
pool3_RNA500
B
Read depth
Variant read count
Variant rank-order
C
Library skew
Cumulative read fraction
Sorted variant fraction
Perfectly uniform
pool1_DNA Gini: 0.141
pool1_RNA1500 Gini: 0.156
pool1_RNA250 Gini: 0.160
pool1_RNA500 Gini: 0.157
pool2_DNA Gini: 0.124
pool2_RNA1500 Gini: 0.141
pool2_RNA250 Gini: 0.144
pool2_RNA500 Gini: 0.141
pool3_DNA Gini: 0.189
pool3_RNA1500 Gini: 0.204
pool3_RNA250 Gini: 0.209
pool3_RNA500 Gini: 0.204
E
de-repression
mutations

Figure S5

FIGURE TITLES AND LEGENDS

Figure 1. Selective enrichment of 3'-tRFs compared to full-length tRNAs in mouse and human cell lines.
(A) Diagram of cellular tRF3a (~18 nt) and tRF3b (22 nt) small RNAs derived from mature tRNAs including their post-transcriptional CCA-tail.
(B) Work flow of hydrolysis-based tRNA sequencing and small RNA sequencing from the same samples.
(C) Quantification of full-length tRNAs, tRF3b and tRF3a fragments. Proportion of tRNA, tRF3a, and tRF3b by amino acid isotype, two biological replicates each, in P19 mouse embryonal carcinoma cells.
(D) Relative counts (%) of tRF3a and of tRNA reads that end in CCA. Enrichment of specific isoacceptors is conserved across mouse and human cell lines. mTSC: mouse trophoblast stem cells.
(E) Lysine isoacceptor ratios of tRNAs and tRFs in P19 cells.
(F) Heat map of log2-fold changes (log2FC) of tRF3a and tRF3b relative to their corresponding full-length tRNA. Asterisks (*) mark significant enrichment (log2FC >1, padj. < 0.01).

Figure 2. 3'-tRFs are stabilized by HENMT1-mediated 2'-O methylation.
(A) Small RNAs with 2'-O methylation at their 3'-end enrich during periodate oxidation treatment. Small RNA oxidized (ox) *versus* untreated (control) from mouse testis are shown ranked by their log2 fold change (log2FC) [dataset GSE95218]. White bars correspond to 3'-tRFs that were expressed but did not exhibit statistically significant changes. For miRNA and piRNA, only a few example sequences are shown.
(B) Volcano plot showing enrichment of mouse testis small RNAs after oxidation treatment. Each dot represents one sequence (piRNA, miRNA) or isoacceptor (3'-tRFs). Significantly enriched or depleted (Log2FC > |1| and padj. < 0.01) piRNAs and 3'-tRFs are colored in taube and red, respectively.
(C) Enrichment/depletion of oxidized samples *versus* control plotted against normalized expression of small RNAs.
(D) Size distribution of piRNAs (upper panel) and 3'-tRFs (lower panel) in *Henmt1*$^{em1Pdz/em1Pdz}$ (henceforth abbreviated *Henmt1*$^{em1/em1}$) and wildtype mouse primary spermatocytes [dataset Gainetdinov et al. 2021].
(E) Volcano plot showing depletion of 3'-tRFs in *Henmt1*$^{em1/em1}$ compared to wildtype primary spermatocytes. Each dot represents one sequence (piRNA, miRNA) or isoacceptor (3'-tRFs). Significantly enriched or depleted (Log2FC > |1| and padj. < 0.01) piRNAs and 3'-tRFs are colored in taube and red, respectively.
(F) Enrichment/depletion in *Henmt1*$^{em1/em1}$ *versus* wildtype (WT) primary spermatocytes plotted against normalized expression of small RNAs.

Figure 3. In the absence of HENMT1, 3'-tRFs are tailed by the terminal nucleotidyltransferases TENT2 and TUT4.
(A) In male germ cells of *Henmt1*$^{em1/em1}$ mice, 3'-tRFs accumulate tails of non-templated nucleotides. Shown is tail length for small RNA classes in wildtype (WT) primary spermatocytes and loss of function mutants for *Henmt1* and the 3'-exonuclease *Plndc1* [data Gainetdinov et al. 2021]. Relates to Figure S3D.
(B) Nucleotide composition of untemplated 3'-nucleotides in wildtype and loss of function male mouse germ cells [data Gainetdinov et al. 2021]. Spermatogonia (spg), primary spermatocytes (spI), secondary spermatocytes (spII), round spermatids (rs).

(C) Adenosine and **(D)** uridine tail length of AGO2-bound small RNAs in HEK293T wildtype (WT) and knockout cells lacking the terminal nucleotidyltransferases TUT4, TUT7, or TENT2 [data Yang et al. 2020]. TKO: triple knockout
(E) Frequency of untemplated nucleotides by length for AGO2-bound small RNAs in HEK293T cells. Frequency was defined as number of miRNA or number of isodecoder tRF3a/b with greater than 10 reads per million relative to number of untailed small RNAs.
(F) Overall tail length in AGO2-bound wildtype (WT) *versus* TUT4/TUT7/TENT2 (TKO) HEK293T cells relative to length of the small RNA without untemplated tail.

Figure 4. HENMT1 restricts LTR-retrotransposition.
(A) Plasmid-based retrotransposition assay: The gene products of *Mus musculus particle type D* (MusD) reverse-transcribe the retroviral RNA of MusD and *Early Transposon* (ETn) and integrate their cDNA into the host genome. The reporter (REP) gene becomes active only after splicing, reverse transcription and integration into the genome. Gag: group-specific antigen, pr: protease; pol: polyprotein containing reverse transcriptase/ribonuclease H and integrase domains; PBS: tRNA primer binding site
(B) HeLa cells are transfected with plasmids, selected for antibiotic resistance (reporter gene) and retrotransposition is quantified (number of resistant cell colonies).
(C) Domain structure of human HENMT1 with the E132A mutation indicated which disrupts methyltransferase (MTase) activity (Peng et al., 2018).
(D) Retrotransposition assay in a clonal Henmt1 CRISPR KO HeLa cell line (clone #29) with expression of functional, wildtype HENMT1 or the catalytic dead HENMT1-E132A mutant, respectively. Loss of HENMT1 methyltransferase activity increased MusD-mediated ETn retrotransposition dependent on the MusD PBS sequence, the target site for 3'-tRFs. Values are mean of colony counts +/- standard deviation.
(E) Small RNA quantitative PCR (*Taqman miRNA Advanced assay*, Thermo Fisher Scientific) with custom primer-probes to detect Lys^3-tRF3b for samples shown in (D). Relative expression was normalized to miR-191-5p. Values are mean expression +/- standard error.

Figure 5. Massively parallel reporter assay (MPRA) to determine 3'-tRF target site rules.
(A) tRF3b target sequences in the mouse genome (mm10) allowing up to three mismatches. tRF3b sequences aligning antisense to the feature annotation are categorized by repeats (UCSC rmsk repeat mask), Gencode transcripts and Gencode transcripts with repeat overlap. "Other" includes alignments to either genomic strand.
(B) $Lysine^3$-TTT tRF3b target sequences in the mouse genome categorized as in (A).
(C) Repeat sequences in (B) split up into transposon families that have $Lysine^3$-TTT tRF3b target sites. All listed families except "other" are of the ERV-K (Lysine) superfamily. MusD6 is an ETnERV2-int retroelement and MMETN-int retroelements include ETnIIbeta used in retrotransposition assays in this study.
(D) Experimental scheme of the MPRA to test the effect of ~1,700 PBS sequences in the MusD 5'-UTR on RNA expression. Sequences were bulk cloned into an expression vector with the MusD6 5'-UTR preceding a reporter gene in place of the Gag open reading frame of the retroelement. After transfection of plasmid pools, cells were harvested to isolate RNA and expression was determined by targeted RNA-seq normalized to DNA abundances in the input plasmid pool.
(E) Ranked, normalized RNA expression of biological replicates including three independent plasmid pools, each transfected at 250 ng, 500 ng, and 1500 ng, respectively, for a total of nine replicates. De-repression is relative to the reference "canonical" PBS sequence (bold sequence in panel J). Seed, central and 3'-region are defined in respect to commonly used miRNA nomenclature and depicted in panel J. Each dot (grey) represents one PBS sequence. Black dots are PBS sequences with mutations in the seed (left panel) or 3'-region (right panel).

(F) The same data as in (E) but grouped by whether mutations in the PBS motif were in seed, central, or 3'-region. Whiskers represent minimum and maximum datapoints less than 1.5x the interquartile range above or below the quartiles all outliers are plotted.
(G) Observed de-repression dependent on the number of mismatches grouped by location within the PBS target site for the library reference sequence. Stars indicate significant de-repression (Benjamini-Hochberg-adjusted $p<0.05$, 1-sample t-test). Lines represent median and quartile values and all data points are plotted.
(H) MAVE-NN latent phenotype (DF) map with low (blue) to high (red) expression. Nucleotides of the reference PBS sequence are marked by dots. Positions are aligned with the sequence overview of genomic MusD elements in (J).
(I) Effect of mutations in the PBS sequence in sliding windows of 3 nt on expression, i.e., de-repression compared to the reference PBS. Positions are aligned with the sequence overview of genomic MusD elements in (J). Lines represent median and quartile values and all data points are plotted.
(J) The PBS sequences of full-length MusD retroelements (5' to 3') in the mouse genome were aligned and ranked by their copy number (indicated on the left). The PBS sequence of MusD6 is the most abundant with thirty-three (33x) copies in the genome. The 3'-end of the Lys^3 primer tRNA and the tRF3b fragment (3' to 5') is depicted on top of the alignment and mismatches in the PBS are shaded grey. The canonical reference PBS sequence (bold) is perfectly complementary to the Lys^3-TTT sequence except for the conserved mismatch at the m^1A position (underlined adenosine). Seed, central and 3'-region were defined as indicated. Nucleotide positions that did not affect replication and priming by Lys^3-tRNA for HIV-1 in (Wang et al., 2018) are colored blue. Asterisks indicate the PBS sequence of MusD elements that are known to actively retrotranspose (Ribet et al., 2004).

Figure S1. 3'-tRFs and full-length tRNAs in mouse and human cell lines
(A) Optimization of alkaline hydrolysis conditions for tRNA hydrolysis sequencing (Gogakos et al., 2017)
(B) Relates to Figure 1C: Quantification of full-length tRNAs, tRF3b and tRF3a fragments. Proportion of tRNA, tRF-3a, and tRF-3b by amino acid isotype in HeLa cells (two biological replicates) and mTSC (four biological replicates).
(C) Proportion of tRNA, tRF-3a, and tRF-3b isoacceptor sequences in all three cell lines (P19, mTSC, HeLa) and their biological replicates.
(D) tRNA, tRF-3a, and tRF-3b expression in percent of total reads clustered by similarity for all cell lines.
(E) Relates to Figure 1F: Heat map of log2-fold changes (log2FC) of tRF3a and tRF3b relative to their corresponding full-length tRNAs for all isodecoder sequences. Asterisks (*) mark significant enrichment (log2FC >1, adjusted p-value < 0.01).
(F) Proportion of Lysine isodecoder tRNA, tRF-3a, and tRF-3b sequences for all three cell lines (P19, mTSC, HeLa). Lys-TTT-3 (yellow) also referred to as Lys^3 is the primer tRNA for MusD/ETn.
(G) Phylogenetic tree of human Lysine tRNA isodecoder sequences and Clustal Omega alignment of the 3'-end of cytoplasmic isodecoder sequences.
(H) Phylogenetic tree of mouse Lysine tRNA isodecoder sequences and Clustal Omega alignment of the 3'-end of cytoplasmic isodecoder sequences.

Figure S2. 3'-tRF analysis of small RNAs from male germ cells of *Henmt1*$^{em1/em1}$, *Pnldc1*$^{em1/em1}$, and wildtype mice (Gainetdinov et al., 2021)

(A) Cartoon of male germ cell types that were FACS-sorted for RNA isolation and small RNA sequencing data produced by (Gainetdinov et al., 2021). Illustration modified from (Gainetdinov et al., 2018).
(B) Relates to Figure 2E: Volcano plot showing 3'-tRFs log2 fold changes (log2FC) in *Henmt1*$^{em1/em1}$ and *Pnldc1*$^{em1/em1}$ compared to wildtype. Colored dots indicate significantly enriched or depleted small RNAs (Log2FC > |1| and padj. < 0.01). Each dot represents one sequence (piRNA, miRNA) or isoacceptor (tRFs).
(C) Relates to Figure 2D: Size distribution of 3'-tRFs in mouse male germ cells as abbreviated in (A) of loss of function mutants for *Henmt1* and the 3'-exonuclease *Pldnc1*.
(D) Relates to Figure 2D: Size distribution of piRNAs in mouse male germ cells as abbreviated in (A) of loss of function mutants for *Henmt1* and the 3'-exonuclease *Pldnc1*.

Figure S3. HENMT1 expression outside the germline and 3'-tRF untemplated nucleotide tailing
(A) *HENMT1* RNA expression in human GTex tissue samples.
(B) Western blot of lysates from HEK293T and HeLa cells probing for HENMT1 expression.
(C) DepMap 'Gene Effect' score of HENMT1 knockdown (RNAi) and knockout (CRISPR)
(D) Length of untemplated 3'-nucleotide tails in germ cell types of *Henmt1*$^{em1/em1}$ *Pnldc1*$^{em1/em1}$, and wildtype mice [data Gainetdinov et al. 2021]
(E) Uridine and adenosine tail length in AGO immunoprecipitated (IP) and total RNA (designated "WT") of HEK293T cells. DKO: double knockout of TUT4 and TUT7; AGO1-Endo: pulldown of endogenous AGO1; AGO2-NEB: pulldown of AGO2 with New England Biolabs (NEB) antibody; AGO1/AGO2-Flag: FLAG-tagged, overexpressed AGO1/AGO2
(F) Uridine and (G) adenosine tail length of AGO2-bound Ala-CGC and Lys-TTT isoacceptor sequences in wildtype (WT) and nucleotidyltransferase knockout in HEK293T cells. DKO: double knockout of TUT4 and TUT7; TKO: TUT4/TUT7/TENT2 triple-knockout

Figure S4. Retrotransposition and small RNA expression during HENMT1 knockdown and in HENMT1 ko clonal cell lines.
(A) Plasmid-based retrotransposition assay during HENMT1 knockdown using *onTarget-plus siRNAs* (Dharmacon).
(B) Luciferase expression with the 5'-UTR of the MusD wildtype and PBS-mutated reporter constructs (Schorn et al., 2017) during knockdown of HENMT1. The luciferase ORF is exactly in place of the first MusD ORF. RLU, relative light units.
(C) Western Blot validation of HENMT1 expression in CRISPR-Cas9 HeLa cells using different guide RNAs (gRNA). Guides targeting the human Rosa (hRosa) locus were used as a negative control. All guides except gRNA #1 showed decrease of HENMT1 expression. Protein amounts were estimated probing the same membrane for GAPDH expression.
(D) Sanger-sequencing traces of genomic DNA isolated from HeLa-Cas9 *HENMT1*-g2 clonal cell lines 29 and 37 was resolved by CRISPR-ID (Dehairs et al., 2016) to show mutations in all alleles of polyploid cells. The resulting amino acid sequences and premature termination (*) are indicated. Guide 2 targets between Cys-56 and Arg-63, at the beginning of the methyltransferase domain. Numbers in brackets indicate amino acid position.
(E) Small RNA quantitative PCR (*Taqman miRNA Advanced assay*, Thermo Fisher Scientific) with custom primer-probes to detect Lys^{3}-tRF3b in HeLa-Cas9 wildtype progenitor cells and clonal Henmt1 knockout (ko) cells. Relative expression was normalized to miR-191-5p. Values are mean expression +/- standard error.
(F) Retrotransposition assays in clonal *HENMT1* CRISPR ko HeLa cell lines, clone #29 and #37, with transient expression of functional, wildtype *HENMT1* or the catalytic mutant *HENMT1-E132A* in a pcDNA3.1 vector backbone. Depicted are colony counts when using different amounts of MusD wildtype or PBS^{mut} plus ETn-reporter plasmid. Loss of HENMT1

methyltransferase activity increased MusD-mediated ETn retrotransposition dependent on the wildtype MusD PBS sequence. Values are the mean of colony counts +/- standard deviation.

Figure 5S. Annotation of MusD full-length elements and validation of MPRA
(A) Replicate correspondence plots. Each point represents the log10 fold change between RNA and DNA abundance of an individual MPRA sequence as measured in two different MPRA replicates. Grey dotted lines indicate equality. Blue dashed lines represented a linear fit of the relationship between the observed values in the two replicates. Pearson R values representing correlation between that pair of replicates is included in each panel. Histograms represent the distribution of fitness values observed in each replicate.
(B) Zipf plot for diagnosing over or under abundant sequences in the MPRA libraries. Each point is the number of reads observed for one MPRA sequence sorted in descending order of read count. Most sequences have similar numbers of read counts but a small number have very high or very low count numbers.
(C) Lorenz plots for diagnosing inequality in library read counts. Each point the fraction of total library reads accounted for by MPRA sequences in the bottom x fraction of the library (when sorted in ascending order by read count). The black line indicates what a library with perfectly uniform read counts would look like. Deviations from perfect equality are quantified using the Gini index which represents the fractional difference in the area under each library Lorenz curve and the uniform Lorenz curve. Values close to 0 are highly uniform and values close to 1 are highly skewed.
(D) Plots show RNA expression of select luciferase reporter plasmids quantified in the MPRA and their protein expression quantified by luciferase assays and expressed in relative light units (RLU). Expression was normalized to the values of the canonical reference PBS sequence described in the main text (Figure 5).
(E) Observed de-repression dependent on the number of mutations relative to the canonical, reference PBS sequence. Stars indicate significant de-repression (Benjamini-Hochberg-adjusted $p<0.05$, 1-sample t-test). Lines represent median and quartiles and all data points are plotted.

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MATERIAL AND METHODS

Cell Lines and Tissue Culture

Mouse trophoblast stem cells (TS6.5 derived from B5/EGFP transgenic mice, kind gift from the Janet Rossant laboratory, The Hospital for Sick Children, Toronto, Canada) were grown in MEF-conditioned medium supplemented with FGF4 (R&D systems, 235-F4-025) as previously described (Tanaka et al., 1998). P19 mouse embryonic teratocarcinoma cells (ATCC, #CRL-1825) were grown in *Alpha-MEM* (Thermo Fisher Scientific, #12571089) with 7.5% bovine calf serum and 2.5% fetal bovine serum (FBS). HeLa cells (ATCC, #CCL-2) were grown in *DMEM* with 10% FBS. All cell lines were grown at 37C and 5% CO_2.

Hydrolysis-based tRNA-sequencing and Small RNA sequencing

RNA was extracted using *TRIzol* (Thermo Fisher Scientific, #15596026) following the manufacturer's instructions but with 80% v/v ethanol washes during precipitation. 20 ug total RNA per sample were separated on a 6% *Novex TBE-Urea Gel* (Thermo Fisher Scientific, #EC68655BOX) and stained with *SYBR Gold* (Thermo Fisher Scientific, #S11494) to excise tRNAs (visible band ~72 nt) and small RNAs (11-40 nt, using oligonucleotide markers). RNAs were eluted in 0.3 M NaCl, 0.5 mM EDTA overnight at 4C, ethanol-precipitated in the presence of *GlycoBlue* (Thermo Fisher Scientific, # AM9516), resuspended in a small volume of water and stored in *DNA LoBind Tubes* (Eppendorf, #022431021). The tRNA fraction was treated as previously described (Gogakos et al., 2017). Briefly, 250 ng of size-selected tRNA in 125 ul water was combined with 125 ul of 20 mM carbonate-bicarbonate buffer pH 9.8 (Na_2CO_3/$NaHCO_3$), and RNA was hydrolyzed for 45 min at 90C. After ethanol-precipitation, RNA was treated with *Calf Intestinal Phosphatase* (NEB, #M0290S) for 1h at 37C to remove phosphate groups at the 3'-end of hydrolysis products. The reaction was stopped by acid phenol-chloroform (Thermo Fisher Scientific, #AM9720) extraction, followed by ethanol-precipitation and several washes with ice-cold 80% v/v ethanol to remove excess salt. After resuspension in RNase-free water, RNA was phosphorylated using *T4 Polynucleotide Kinase* (NEB, #M0201S) and cleaned up with acid phenol-chloroform followed by ethanol precipitation before it was used as input in a small RNA library preparation. Small RNA library preparation was performed on both, the treated tRNA fraction and the small RNA fraction of the same samples, using 3' and 5' $(N)_4$-randomized adapters and PEG-8000 according to *Supplementary Protocol 4* of Giraldez et al. 2018 (Giraldez et al., 2018).

Analysis of hydrolysis-based tRNA-sequencing and 3'-tRFs

Reads were quality filtered using Gordon Assaf's *fastx-toolkit*. *Cutadapt* (Martin, 2011) was used to clip adapter sequences and size select for 14-60 nt read length. Reads that end in the post-transcriptional CCA-trinucleotide tail of mature tRNA were used for analysis of both, (i) small RNA data, specifically tRF3a and tRF3b fragments ending in CCA, as well as (ii) hydrolysis-based tRNA data, since these CCA-ending reads directly correspond to the amount of full-length parental tRNA of a given isoacceptor or isodecoder. Two alignment strategies were used. The first approach used the repeat mask annotation of the human and mouse genome, respectively

(ftp://hgdownload.cse.ucsc.edu/goldenpath/hg38/database/rmskOutCurrent.txt.gz, ftp://hgdownload.soe.ucsc.edu/goldenPath/mm10/database/rmskOutCurrent.txt.gz). All reads ending in CCA were clipped for this trinucleotide using *Cutadapt* (Martin, 2011) and aligned to the human or mouse genome (UCSC hg38 and mm10, respectively) that has been masked for everything except sequences annotated as tRNA in the repeat mask. *Bowtie2* was used to randomly distribute multi-mapping reads (bowtie2 -N 1 -L 15 --gbar 20 -R10) (Langmead and Salzberg, 2012). Reads with up to two mismatches were processed for downstream analysis. Details of this approach are described in (Schorn et al., 2017). This is the most inclusive approach that preserves all and any tRNA-derived sequences and their genomic loci. Data displayed in Figure 1C was generated by the first approach. Differential expression analysis and all other figures used read counts obtained by the second approach. The second approach used the UCSC *GtRNAdb* annotation (https://gtrnadb.ucsc.edu/genomes/eukaryota/) for mouse (Mmusc10) and human (Hsapi38), respectively, which contains isodecoder identity. The post-transcriptional CCA-tail was added to the 3'-end of the mature tRNA sequences in fasta format and used to build a *Bowtie* index (Langmead et al., 2009). Reads ending in CCA were directly aligned to this index allowing up to two mismatches to account for RNA modifications (bowtie -f --best --tryhard -v 2). Differential expression between 3'-tRF and hydrolysis-based tRNA read counts was calculated using *DESeq2* (Love et al., 2014). tRF3a fragments were defined as 17-19 nt in length and tRF3b fragments were required to be 22 nt long. Analysis of Lysine isodecoder tRNA sequences (Figure S1F, G) was performed using the same UCSC *GtRNAdb* mature tRNA sequence annotation. Lysine isodecoder tRNA sequences for mouse and human, respectively, were aligned in *Jalview* (version 2.11.4.1) using the *Clustal-Omega* algorithm and default parameters. The *Clustal-Omega* alignment and its distance matrix was used to make a phylogenetic tree in *Jalview* using the neighbor joining method.

<u>Analysis of small RNA from male mouse germ cells and total testis</u>

Mouse testis small RNAs treated with $NaIO_4$ were analyzed for enrichment compared to untreated samples (GSE95218) (Yang et al., 2019b). Sorted, male germ cells from wildtype, *Henmt1*$^{em1Pdz/em1Pdz}$, and *Pnldc1*$^{em1Pdz/em1Pdz}$ (also referred to as *Henmt1*$^{em1/em1}$ and *Pnldc1*$^{em1/em1}$) (PRJNA660633) (Gainetdinov et al., 2021) were analyzed for depletion of small RNA classes. Analysis of tRNA-fragments was done as described in Schorn et al. 2017 (Schorn et al., 2017). Briefly, reads were split into sequences that end in CCA and reads that don't. Reads that end in CCA were clipped for their terminal CCA and aligned to the UCSC mm10 genome with everything masked that is not a tRNA in the UCSC rmsk annotation (see previous section). Multi-mapping reads were distributed randomly using Bowtie2 (bowtie2 -N 1 -L 15 --gbar 20 -p2 -f -R10) (Langmead and Salzberg, 2012). Using Bamtools (http://github.com/pezmaster31/bamtools), sequences were filtered for 0-2 mismatches to allow for RNA modifications common in tRFs. For reads that matched these criteria, tRNA codon, genomic coordinates and read counts were collected, asking for an original read size of 17-19nt (tRF3a) and 20-23 nt (tRF3b). For downstream sequence and size distribution analysis, CCA-tails were added back.

Reads that did not end in CCA were directly mapped to the genome with all but tRNA sequences masked as described above. tRFs that span splice-sites are not captured by this approach. Again, tRNA codon, loci and read counts were collected for downstream analysis.
Reads that did not map tRNA loci were collected for further analysis of other small RNA classes and intersection with repeats. Reads that aligned to a hairpin precursor (miRBase version 22) (Kozomara et al., 2019) with 1 mismatch were classified as miRNAs. Reads that did not match any previous category, were aligned to the UCSC mm10 mouse genome using *Bowtie2* (bowtie2 -N 1 -L 15 --gbar 20 -p2 -f -R10) distributing multi-mappers randomly, and intersected with the current USCS *rmsk* repeat annotation using *BEDtools* to determine repeat- and transposon derived small RNA (Langmead and Salzberg, 2012; Quinlan and Hall, 2010). Subsequently, reads were intersected with the piRNA annotation piRNAdb.mmu.v1.7.6 (https://www.pirnadb.org) (Rosenkranz et al., 2022) asking for at least >90% overlap. To avoid over-counting when collapsing on piRNA names (several previously annotated piRNAs can overlap one read), *BEDtools grouby* was used (bedtools groupby -grp 1-4 -c 5 -o collapse).
The following raw read counts were collected and used for *DESeq2* analysis to determine significantly enriched or depleted small RNAs: 3'-tRFs, other tRFs (sequences that do not end in CCA but map to genomic tRNAs), miRNAs and piRNAs with or without repeat-overlap. To obtain size distributions, reads normalized to spike-in calibrators per number of cells were plotted by length.

Analysis of untemplated, 3'-end nucleotide tails of small RNAs

Analysis of 3'-tailing of small RNAs utilized data from male germ cells of wildtype, *Henmt1*$^{em1Pdz/em1Pdz}$, and *Pnldc1*$^{em1Pdz/em1Pdz}$ mice (PRJNA660633) (Gainetdinov et al., 2021), as well as of HEK293T cells with loss of function mutations in the terminal nucleotidyltransferase genes TUT4, TUT7, and TENT2 (GSE183384) (Yang et al., 2022). Small RNAs were mapped to an index of *miRBase22* mature miRNAs (Kozomara et al., 2019) and the last 22 nt of mature tRNA sequences including their CCA-tail (generated from UCSC *GtRNAdb* as described above). The m^1A RNA modification in 3'-tRFs often leads to misincorporation of nucleotides during the reverse transcription step of sequencing protocols. To allow alignment to the index without mismatches, this position was replaced by degenerate nucleotides (A/G/T/C). To analyze non-templated tailing of small RNAs, an iterative approach was used: initially, all reads were mapped to the index and those that aligned with zero mismatches were counted as total, untailed small RNA. Reads that did not align, including those with a tail, were shortened by exactly one nucleotide at their 3' end, remapped, and counted as a tail length +1 nt if they aligned with no mismatches in this iteration. This process was repeated seven times, each time recording the nucleotide that was clipped from the 3' end to keep track of tail composition. I.e., tail length reflects the number of iterations that were required to map a read.

CRISPR-knockout and rescue of HENMT1

The puromycin resistance gene in pLenti-Cas9-P2A-puro (Addgene #110837) was replaced by the zeocin resistance gene of pcDNA3.1(+)-zeo. Lentiviral particles carrying Cas9-zeo were harvested from HEK293T cells (ATCC #CRL-3216) after calcium phosphate transfection of

pLenti-Cas9-P2A-zeo, pCMV-VSV-G (Addgene #8454), psPAX2 (Addgene #12260 Didier Trono lab). HeLa target cells were infected at low MOI and selected for zeocin to produce Cas9-expressing stock cells. CRISPR guide RNAs (gRNAs) against *HENMT1* were designed using the CSHL domain-focused algorithm (https://mojica.cshl.edu/) and cloned into LR2.1T-puro (kind gift of Chris Vakoc lab, CSHL), a derivative of LRG2.1-Puro (Addgene Plasmid #125594). All gRNA sequences are listed in Supplemental Table S1. HeLa-Cas9 target cells were infected with lentivirus carrying gRNAs to induce targeted mutations and harvested for Western Blot with HENMT1 polyclonal antibodies (Thermo Fisher, Catalog # PA5-55866, rabbit polyclonal, antigen sequence: MEENNLQCSS VVDGNFEEVP RETAIQFKPP LYRQRYQFVK NLVDQHEPKK VADLGCGDTS LLRLLKVNPC IELLVGV, corresponding to N terminal amino acids 1-77 of human HENMT1). To generate stable clonal cell lines, HeLa cells were infected at low MOI to express hHENMT1_gRNA_#2 (Supplemental Table S1) and selected for puromycin resistance. ~40 colonies were hand-picked to expand clonal cell lines for cryo-preservation and to collect protein lysate for HENMT1 Western detection. Genomic DNA was extracted from candidate clones (Invitrogen PureLink Genomic DNA Mini Kit, #K182001) and CRISPR-induced mutations were confirmed by Sanger sequencing of PCR-amplified genomic DNA (primers hHenmt1_gDNA_g2_#1/#2) of ~300 bp around predicted gRNA target sites. Sanger sequencing traces of multiple alleles were resolved (Fig S4D) using the 'CRISP-ID' algorithm (Dehairs et al., 2016).

HENMT1 cDNA (NM001102592) was purchased from *Transomics* and cloned into the pcDNA3.1(+)-neo backbone (Thermo Fisher Scientific, #V79020) for transient expression. The cDNA was mutated to be resistant to CRISPR gRNA cleavage, but without changing the amino acid sequence of HENMT1. Catalytic point mutations were introduced by primer-mediated PCR mutagenesis using high fidelity polymerases (Phusion High-Fidelity DNA Polymerase, NEB #M0530L and *KOD Xtreme Hot Start DNA Polymerase* Sigma-Aldrich, #71975-M) and subsequent DpnI digest. All DNA amplified by PCR or assembled using *NEBuilder HiFi* (NEB #E5520) was confirmed by Sanger-sequencing or *Plasmidsaurus* high coverage NGS sequencing service. Varying amounts of *HENMT1* expressing plasmids were transfected and proteins were checked by Western Blot for their expected molecular weight. For functional rescue of HENMT1, 900 ng pcDNA3.1(+)-neo-*HENMT1* plasmids (see previous section) per 12-well were co-transfected with transposon plasmids.

Transposition assays

The neomycin resistance cassette in pCMV-ETnIIbeta-TNFneo (Ribet et al., 2004) that contained a mirtron was replaced by a SV40-driven blasticidin resistance gene with Tk-poly(A) interrupted by a human globin intron (M91037.1) in reverse orientation (amplified from pJJ101-L1.3-blast, kind gift of the John Moran laboratory, University of Michigan). For each transposition assay, 1.5 x 10^5 HeLa cells were seeded in triplicates in 12-well plates the day before transfection. 20 ng pCMV-MusD6 (Ribet et al., 2004) and 200 ng pCMV-ETnIIbeta-blast plasmids per 12-well were co-transfected using *Lipofectamine2000* (Thermo Fisher Scientific, #11668019). Transposon sequence accession numbers are AC124426 and AC126548, respectively. ETnIIbeta is most closely related to the MMETn-int consensus sequence of the *RepeatMasker* annotation; MusD6

is an ETnERV2-int element. 1-2 days post transfection, cells were transferred onto 10 cm plates and put under blasticidin selection starting day 5-6 until complete. Blasticidin-resistant colonies were fixed with 10% v/v formaldehyde in PBS, stained with 0.1% w/v methylene blue in PBS, and counted (each colony = one retrotransposition event).

siRNA-mediated knockdown of HENMT1

For HENMT1 knockdown experiments, *ON-TARGETplus Human HENMT1 siRNA SMART pool* (Dharmacon, # L-016309-02) were co-transfected with the above-described transposon plasmids to a final concentration of 25 nM. A 1:1 mixture of *ON-TARGETplus Non-targeting Control siRNAs* #2 (Dharmacon, #D-001810-02) and #3 (Dharmacon, #D-001810-03) was used as negative control. Oligonucleotide sequences are listed in Supplemental Table S1.

Quantitative PCR RNA analysis

RNA was extracted using *TRIzol* (Thermo Fisher Scientific, #15596026) following the manufacturer's instructions but with 80% v/v ethanol washes during precipitation. Total RNA was treated with *TURBO DNaseI* (Thermo Fisher Scientific, #AM1907). Reactions were stopped by heat inactivation after addition of EDTA and cleaned up using *AMPure XP beads* (Beckman Coulter, #A63880). For long RNA quantification, RNA was reverse transcribed using *SuperscriptIII* (Thermo Fisher Scientific #18080044) following the manufacturer's instructions and quantified using *PowerTrack SYBR Green Master Mix* (Thermo Fisher Scientific, #A46109). Reactions were run on a *QuantStudio 6 Flex Real-Time PCR System* (Thermo Fisher Scientific). For small RNA quantification, reverse transcription was performed using *TaqMan Advanced miRNA cDNA Synthesis Kit* (Thermo Fisher Scientific, #A28007) and *TaqMan Fast Advanced Master Mix* (Thermo Fisher Scientific, #4444556), following the manufacturer's protocol. A total of 10 ng of purified RNA was used for cDNA synthesis, which included polyadenylation, adapter ligation, reverse transcription, and miR-Amp pre-amplification steps. Quantitative PCR was carried out on a *QuantStudio 6 Flex Real-Time PCR System* (Thermo Fisher Scientific) using custom-designed *TaqMan Advanced miRNA Assays* (Thermo Fisher Scientific, #A25576) specific for Lys-tRF3b (UCA AGU CCC UGU UCG GGC GCC A). C_t values were normalized to the endogenous control miRNA, miR-191-5p, using the ΔΔCt method. All primer and probe sequences are listed in Supplemental Table S1.

Genome-wide 3'-tRF target site analysis

Mature tRNA sequences of the mm10 mouse genome annotation were downloaded from the UCSC table browser ('Genes and Gene Predictions'/'tRNA genes'). After adding a CCA-trinucleotide for the post-transcriptional 3'-tail, the last 22 nt were used to generate a fasta file of tRF3b fragments. Sequences were aligned against the UCSC mm10 genome allowing up to three mismatches (bowtie -f --all -v 3) (Langmead et al., 2009). Aligned sequences were intersected with transcript (Gencode version M25) or repeat annotations (ftp://hgdownload.soe.ucsc.edu/goldenPath/mm10/database/rmskOutCurrent.txt.gz) using BEDTools (intersectBed -wb -f 1.0) (Quinlan and Hall, 2010). The sequence of the 22 nt tRF3b fragment derived from the Lysine^3-TTT isodecoder is 5'-TCAAGTCCCTGTTCGGGCGCCA-3'

and its target sequences are the reverse complement. In other words, target sites in transcripts or repeat-derived RNA are defined by tRF3b alignments on the opposite strand.

Annotation of MusD full-length, genomic copies

All ERVK:ETnERV-int entries larger than 6 kb were collected from the current UCSC Golden Path repeat mask annotation (see above) as putative MusD full-length elements. Using *BEDTools* closest, upstream and downstream annotations on the same strand were collected to identify flanking LTR sequences and related sequences of the ETn superfamily that are contained between their flanking LTRs. Primer binding site (PBS) sequences were identified by a genome-wide alignment of tRF3a sequences (analogous to above described tRF3b alignments) and required to be immediately downstream of the 5'-LTR of > 6 kb elements. PBS coordinates were extended for 4 nt downstream using *BEDTools* (bedtools slop -s -l 4 -r 0) and converted to fasta format to collapse and count occurrences (Figure 5J). 71 elements matched all the above criteria. 65 are flanked by ERVK:ERVB7_1-LTR_MM, two by ERVK:ERVB7_2-LTR_MM, two by ERVK:ERVB7_2B-LTR_MM, and one each by ERVK:RLTR9E and ERVB7_4-LTR_MM. NCBI *ORFfinder* and *orfipy* (Singh and Wurtele, 2021) were used to find candidate open reading frames (ORFs) for all assembled MusD elements. 8 elements had all three retroviral ORFs (Gag 593 aa, Pr 318 aa, Pol 868 aa); the remaining elements had either truncated Pr or Pol ORFs. A total of 16 elements had intact Gag ORFs and hence are potentially protein-coding MusD elements with a target site for Lysine^{3}-TTT 3'-tRFs in their 5'-UTR.

MPRA experimental design and library construction

A massively parallel reporter assay (MPRA) was employed to test the effect of the tRNA primer binding site (PBS) sequence on MusD6 transposon expression. A pool of ~1,700 single-stranded DNA oligos (*Twist Biosciences*) containing mutations at the PBS site and 4 nt downstream was cloned into pGL4.21-MusD6 (Schorn et al., 2017). In brief, the oligo pool was amplified using primer MPRA_#1 and MPRA_#2 (Supplemental Table S1), *KAPA HiFi HotStart Polymerase* (Roche, # KK2502), and low cycle numbers to maintain sequence complexity and generate double-stranded DNA. The resulting PCR product was purified using *AMPure XP beads* (Beckman Coulter, #A63880) bead-to-sample ratio of 1.8X) and verified for the expected size using the *High Sensitivity DNA Kit* (Agilent #5067-4626). The pGL4.21-MusD6 plasmid backbone was linearized by restriction digest and amplified with *Q5 High Fidelity 2X Master Mix* (NEB, #M0491L) to generate a 20 bp overlap with the insert pool that was bulk cloned into the plasmid backbone using *NEBuilder HiFi DNA Assembly* (NEB #E5520). The assembled plasmid pool was electroporated into *10-beta electrocompetent E. coli* (NEB, #C3020K)) in three independent transformations that resulted in replicate DNA input pools (MPRA DNA pool #1, #2, #3).

HeLa cells were seeded at 2x 10^5 cells HeLa cells per 6-well one day prior to transfection with three different amounts (250 ng, 500 ng, 1500 ng) of DNA pool #1 to #3 using *Lipofectamine2000* (Thermo Fisher Scientific, #11668019), resulting in three (amounts) times three (DNA input pool), a total of nine conditions. One day post transfection, cells were washed with 1x phosphate buffered saline (Thermo Fisher Scientific, #20012050) and RNA was extracted using *TRIzol Reagent* (Thermo Fisher Scientific, #15596026). A total of 5 µg of DNase-treated RNA was

reverse transcribed using *SuperScript IV VILO Master Mix* (Thermo Fisher Scientific, #11756050) which includes both, oligo(dT) and random primers. The resulting cDNA was amplified with *Q5 High Fidelity 2X Master Mix* (NEB, #M0491L) for three cycles using primers MPRA_#3 and MPRA_#4 (Supplemental Table S1) that have complementarity to the Illumina sequencing primers. For input MPRA DNA libraries, 80 ng of template was split across four 50 µl PCR reactions and amplified for three cycles. The resulting PCR products were purified using *AMPure XP beads* (Beckman Coulter, #A63880) at a bead-to-sample ratio of 1.2X. The minimum number of additional PCR cycles for library amplification was determined in a test qPCR using *PowerUp SYBR Green Master Mix* (Thermo Fisher Scientific, #A25743). Libraries were amplified with Illumina-P5-universal-for and Illumina-P7-index-rev primers (Supplemental Table S1), purified using a right-sided size selection with *AMPure XP beads* (Beckman Coulter, #A63880) with an initial bead-to-sample ratio of 0.7X followed by 1.1X, and eluted in *UltraPure Water* (Thermo Fisher Scientific, #10977049). Final libraries were verified for their expected size of ~300 bp (*Agilent High Sensitivity DNA Kit,* Agilent #5067-4626), and quantified (*KAPA Library Quantification Kit Illumina platforms*, Roche #KR0405) before pooling. RNA-libraries (expression) and DNA-libraries (input plasmid pools) were sequenced on an *Illumina NextSeq500 Mid Output* single-end 150bp run.

MPRA analysis

Reads were required to have 100% match with the upstream and downstream 5'-UTR sequence of MusD6 (Genbank AC124426, Ribert et al. 2004): (1) cutadapt -m 77 --error-rate 0 --overlap 48 -a ACGCTGCTTTGGAGCTCCACAGGAAAGGATCTTCGTATCGGACATCGGAGCA; (2) cutadapt --action none -m 48 --error-rate 0 --overlap 51 -g CCTTCTCAGTCGAACCGTTCACGTTGCGAGCTGCTGGCGGCCGCAACATTT. Subsequently, reads were required to align with zero mismatches to an index of the *Twist Biosciences* DNA sequence input pool using *Bowtie* (Langmead et al., 2009). *Bowtie2* (bowtie2 -N 1 -L 4 --rdg 1,1 --mp 2) (Langmead and Salzberg, 2012) and *Samtools* (samtools calmd -e) (Li et al., 2009) were used to generate CIGAR strings and to annotate mismatches and indels compared to the reference sequence. The reference sequence was the canonical PBS with a perfect 18 nt match with its Lysine^3-TTT primer tRNA, extended for 4 more nucleotides of the MusD6 sequence, with the mismatch to m^{1}A underlined: 5'- TGGCGCCCGAACAGGGACCTGA-3'. Downstream analysis in this study only contains sequences without indels. Reads that fulfilled all above criteria were filtered for sequences that have mutations only within the 22 nt tRF3b target site (= 18 nt PBS plus 4 nt downstream), then clipped for all nucleotides upstream and downstream. Read counts were collected for sequences that had a minimum of 100 reads across all three DNA input libraries.

Relative abundances for each sequence in the MPRA library were calculated by dividing the read count of this sequence by the total read count in each condition. The original DNA library used for transfection was sequenced to establish baseline abundances. RNA/DNA fold changes were calculated by dividing RNA abundances by DNA abundances and $\log_{10}$ transformed. De-repression relative to the reference, canonical PBS was calculated by subtracting the $\log_{10}$ fold change of the reference sequence from each variant.

For the analysis of mutational effects by region or sliding window, we selected sequences containing mutations only in the region or sliding window of interest. For regional analysis, sequences were further stratified by mutation count within the region. Regions were defined as follows: "seed" region positions t2-7, "central" region positions t9-13, and "3'-region" positions t14-22. We used a one-sample t-test to assess the significance of mean de-repression relative to wild type. False discovery rates were corrected for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). All statistical analyses were performed using SciPy (Virtanen et al., 2020). For the analysis of expected effects of individual mutations, we used global epistasis regression with an additive genotype to latent phenotype model. This approach accounts for non-linear effects of multiple mutations due to global non-linearities in the measurement process. The analysis was performed using MAVE-NN (Tareen et al., 2022) with default parameters. To fit the global epistasis model, we randomly split the library into training (80%) and validation (20%) sets. The training set was used to fit the model while the validation set was used to evaluate model performance. Additive model parameters have a direct interpretation as the expected effects of mutations on the latent phenotype. However, interpretation of these effects is complicated by gauge freedoms in the model that are not constrained by the data. We resolved these gauge freedoms by constraining parameters corresponding to the canonical reference PBS sequence to zero, effectively using it as a reference point. Thus, parameter values reflect effects on the latent phenotype relative to the canonical PBS sequence.

Luciferase assays

A number of sequences from the MPRA plasmid pool were cloned as single sequences and tested for luciferase expression. Oligos (Supplemental Table S1) were amplified with primers L1/L2 (Supplemental Table S1) by 14 cycles of PCR with *KOD Xtreme Hot Start DNA Polymerase* (Sigma-Aldrich, #71975-M). The resulting dsDNA was purified using *RNAClean XP beads* (Beckman Coulter, #A63987). The pGL4.21-MusD6 plasmid (Schorn et al., 2017) was amplified by 30 cycles of PCR with *KOD Xtreme Hot Start DNA Polymerase* (Sigma-Aldrich, #71975-M) and primers L3/L4 (Supplemental Table S1), and purified by agarose gel extraction (Qiagen). The pool of dsDNA was inserted into the linearized vector by *NEBuilder HiFi DNA Assembly* (NEB #E5520). Individual colonies were picked to isolate DNA and sequence confirmed by Sanger sequencing (primer L5, Supplemental Table S1) and *Plasmidsaurus* whole plasmid sequencing.

50,000 HeLa cells were transfected in 24-well plates with 5 ng of each pGL4.21 *Firefly* luciferase reporter constructs, and 205 ng of pcDNA3.1(+)-neo as filler DNA per well using *Lipofectamine2000* (Thermo Fisher Scientific, #11668019). Cells were lysed 24 h post transfection in 100 ul of Glo Lysis buffer (Promega, #E2661). 40 ul of lysate per sample were used to measure luciferase activity using the *Dual-Glo Luciferase Assay System* (Promega, #E2940) according to the manufacturer's instructions, using a *GloMax Discover Microplate reader* (Promega) at 0.3 sec integration time. Relative light units (RLU) were calculated by normalizing *Firefly* luminescence values to total protein amount determined by Bradford Protein Assay (BioRad, #5000006), measuring absorbance at 595 nm.

Statistical analysis

PRISM version 10.3.1 was used to perform statistical analyses of luciferase and transposition assay results using two-tailed Student's t-test. *R* version 4.4.1 was used for data visualization and statistical analysis of read counts in *DESeq2* (Love et al., 2014). Software for sequence analysis was installed and updated using *Bioconda* (Grüning et al., 2018). For statistical analysis of the massively parallel reporter assay data, see section 'MPRA analysis'.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the Lead Contact, Andrea Schorn (aschorn@cshl.edu).

Data and code availability

Sequencing data have been deposited at *National Center for Biotechnology Information Sequence Read Archive* and are publicly available as of the date of publication using accession numbers GSE296629, GSE296630, and are publicly available as of the date of publication. Original western blotting images have been deposited at *Mendeley* (10.17632/phccbh57wx.1) and are publicly available as of the date of publication. All published software required to reanalyze the data reported in this paper is described in the material and methods section. Code for analyzing MPRA data and producing associated figures can be found at *GitHub* https://github.com/jackdesmarais/TRf_transposon_silencing_MPRA; the release associated with this submission is archived on *Zenodo* at https://zenodo.org/records/15361369 (DOI: 10.5281/zenodo.15361369). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

Supplementary Table S1

category	name	sequence 5'-3'
siRNAs	ON-TARGETplus Human HENMT1 siRNA SMART pool (Dharmacon, Cat# L-016309-02)	GCAGAGACUCCUUGCGUAU UUUCAGGGUCUCAGCGAUA CCGCUUAUGUGCUAAUGAA AGUUUAAACCUCCACUAUA
	ON-TARGETplus Non-targeting Control siRNAs #2 and #3 (Dharmacon, Cat# L-001810-02 (#2), L-001810-03 (#3))	UGGUUUACAUGUUGUGUGA (#2) UAGCGACUAAACACAUCAAUU (#3)
primer, probes	Twist oligos for MPRA	see separate sheet
	L1	CCTTCTCAGTCGAACCGTTCACGTTGCGAGCTGCTGGCGGCCGCAACATTT
	L2	GTACCTGTCCGTTGCTCCGATGTCCGATACGAAGATCCTTTCCTGTGGAGCTCCAAAGCAGCGTTCCTCTTAGCATCTCTCTGCCATTCT
	L3	GGACATCGGAGCAACGGACAGGTACACATG
	L4	CTCGCAACGTGAACGGTTCGACTGAGAAGG
	L5	CGCAAATGGGCGGTAGGCGTG
	MPRA #1	CCTTCTCAGTCGAACCGTTC
	MPRA #2	CATGTGTACCTGTCCGTTGC
	MPRA #3	ACACGACGCTCTTCCGATCTccttctcagtcgaaccgttc
	MPRA #4	GACGTGTGCTCTTCCGATCTcatgtgtacctgtccgttgc
	Illumina-P5-universal-for	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	Illumina-P7-index-rev	CAAGCAGAAGACGGCATACGAGATiiiiiiGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	hHenmt1_gDNA_g2_#1	TGTGAGGGGTGAAAGCTAAGT
	hHenmt1_gDNA_g2_#2	GGTTCAAAATGCAGGCGGT
	TaqMan Advanced miRNA custom assay for Lys-tRF3b, (Thermo Fisher Scientific, Cat# A25576-customized)	proprietary
	TaqMan Advanced miRNA assay for hsa-miR-191-5p, (Thermo Fisher Scientific, Cat# A25576-inventoried)	proprietary
CRISPR guide RNAs	hHENMT1_gRNA_#1	CCACTAAATTTTTAACGAAC
	hHENMT1_gRNA_#2	GTGGTGATACTTCACTCTTA
	hHENMT1_gRNA_#3	GAGGATAAATTACGATGGAG
	hHENMT1_gRNA_#4	ATTCGTTAGCTCCTTTCCTG
	hHENMT1_gRNA_#5	ATGGTCAAATTCAGATCCCG
	hHENMT1_gRNA_#6	ATTGTATCATGGCTCCGTTG
	hHENMT1_gRNA_#7	GAGAGAGACTCTCGTTTGCT
	hHENMT1_gRNA_#8	GAATCTCTTAAGGTCACTGA

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