SUPPLEMENTARY INFORMATION

Regulation of La/SSB-dependent viral gene expression by pre-tRNA 3’ trailer-derived tRNA fragments

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SUPPLEMENTARY FIGURES

Figure S1. Profile of tRFs in Huh7. (A) Length distribution of tRFs (tRFs in tRF_5, tRF_3, and tRF_U3 families) (sky blue) and miRNA species (gray) in Huh7 cells (n = 2; GSM3666020 and GSM3666021). (B) Classification of the tRFs grouped according to their positions on pre-tRNA and lengths. (C) Pie chart representing the proportion of the indicated tRFs in Huh7. Bar charts represent the proportions of the indicated tRFs belonging to the three major families, sorted by length.

—tRFs were classified into five families according to their loci on the tRNA molecule and their lengths (Supplementary Figure S1B). The tRF_5 and tRF_3 family tRFs represent the species carrying the 5'- and 3'-end (bearing a “CCA” non-template addition) sequences of mature tRNAs, respectively, which accounted for 19.6% of tRFs in Huh7 (Supplementary Figure S1C). These families were further grouped according to the length of tRFs: “a” (tRF_5a and tRF_3a), “b” (tRF_5b and tRF_3b), and “c” (tRF_5c and tRF_3c) sub-families, which were 16–21 nt, 22–27 nt, and ≥ 28 nt in length, respectively. The tRF_U3 family tRFs, which represent pre-tRNA 3' trailer sequences (including the trailer species lacking 1 nt from the 5'-end of the predicted trailer sequences), mainly consisted of 17–20-nt-long tRFs (Supplementary Figure S1D). Most tRFs derived from mature tRNAs belonged to the i-tRF family (79.3%), which lacked the mature tRNA 5’- or 3’-end sequences, with over 50% starting 30–36 nt downstream from the mature tRNA 5’-end (Supplementary Figure S1C).
Figure S2. Quantification of pre-tRNA-Ser(TGA), mature tRNA, and tRF_U3_1 copy numbers in a single Huh7 cell. (A–C) Total RNA (20 µg) from Huh7 was analyzed by northern blotting using the indicated probes to determine the copy number of pre-tRNA-Ser(TGA), mature tRNA, and tRF_U3_1. In (A and B), known copy numbers of synthetic tRF_U3_1 (19-nt) and/or in vitro transcribed (IVT) pre-tRNA-Ser(TGA) (101-nt) were loaded along with total RNA samples and band intensities were quantified by phosphorimager analysis. Of note, pre-tRNA gave an approximately 2-fold higher band intensity than tRF_U3_1, which is likely due to difference in blotting and/or UV-crosslinking efficiency between the standard molecules. Representative blots of three independent experiment are presented. Shown in (C) are the copy numbers of indicated tRNAs and tRF_U3_1 in a single Huh7 cell. Their copy numbers per cell were determined by assuming that 20.12 pg total RNA is equivalent to one cell, since 100 µg of total RNA was routinely recovered from 4.97 × 10^6 cells. (D and E) Using the probe #1 used to detect mature tRNA throughout the study (unless otherwise specified) and two additional 3′-end probes with different lengths, copy numbers of pre-tRNA and mature tRNA were determined as in (A). Similar specific activities of the radiolabeled probes were verified by autoradiography (D). In (E), three identical RNA sample sets, which were resolved on a 10% denaturing polyacrylamide gel, were blotted together before each set in three separated panels was hybridized with the indicated probes. All blots were processed in parallel under the same conditions. The blots highlighted in sky-blue boxes were used to determine the ratio of mature tRNA to pre-tRNA. pre-tRNA* represents the endogenous pre-tRNA-Ser(TGA) bearing both 5′-end leader and 3′-end trailer, which migrated slightly more slowly than the IVT pre-tRNA lacking the 5′ leader. Error bars are standard deviations of three independent experiments. ***P < 0.001; n.s., not significant by unpaired two-tailed Student’s t-test.
Figure S3. Features of the pre-tRNA 3’ trailer-derived tRFs identified in Huh7 cells. (A) Box-and-whisker plot for the liver cancer cell lines (Huh7 and Huh7-derived cell lines (n = 5); GSE128164 and GSE57381). (B) Detection of tRF_U3_1 in human primary hepatocytes (hPHs) by northern blotting as described in Figure 1D. (C) Northern blot analysis for the selected tRF_U3 family tRFs. Probes specific to the indicated trailers were used for analysis of total RNA (20 µg) from Huh7 and CCD-18Co cells along with the indicated synthetic tRFs (0.1 ng). (D) Box-and-whisker plots show relative levels of the selected miRNAs upregulated in liver cancer cells (NC, normal; C, cancer). (E) Northern blot analysis of total RNA from the indicated subcellular fractions derived from an equal number (4.5 × 10^6) of Huh7 cells. Cellular fractionation was verified by northern blotting for the indicated RNA markers. Nucl, nuclear fraction; Cyto, cytoplasmic fraction. Predominant, albeit not exclusive, cytoplasmic localization of tRF_U3_2 as well as tRF_U3_1 (see also Figure 1F) is contrast to a previous observation of exclusive cytoplasmic accumulation of tRF_U3_1 (also named tRF-1001) (1). It is not yet known whether and how pre-tRNA nuclear export is regulated in different transformed cell lines. (F) U-tail length variance in the top 23 abundant tRF_U3 family tRFs identified in Huh7 cells (see also Supplementary Table S2).
Figure S4. La/SSB preferentially binds to pre-tRNA-Ser(TGA) 3’-end trailer. (A) Identification of the prominent protein band indicated by the arrowhead in Figure 2A as the La/SSB protein (29 matched peptides with 36% coverage and a Mascot score of 876) by LC-MS/MS analysis. Shown in red on the human La/SSB sequence are the identified peptides. -continued next page
(B) Depth plot for La/SSB-crosslinked RNAs [PAR-CLIP assay dataset (GSM2521600) from HEK293] mapped to chr10.tRNA2-Ser(TGA). The reads with at least one T-to-C conversion (solid and dotted red lines) were mainly mapped to the pre-tRNA 3′-end region (nt 65–102). (C) Preferential binding of La/SSB to the trailer 3′-end U-tail. The La/SSB-crosslinked pre-tRNA-Ser(TGA)-derived, RNase A-protected tRFs were sorted according to their starting nt positions. Red arrowhead, RNase A cleavage site; Green arrowhead, RNase Z cleavage site. (D) Depth plot for the five major read groups (> 5% of total in (C)) starting from the indicated positions (+66, +72, +73, +75, and +83 nt) and La/SSB-crosslinked sites (dark blue bar) in each group. The nt positions where at least one T-to-C conversion was detected in > 20% of reads (at least once in the five major groups) are marked by purple circles in (C).
Figure S5. The free trailer tRF tRF_U3_1 binds to La/SSB more efficiently than its precursor. La/SSB was immunoprecipitated from cell lysates of Huh7. RNA in the La/SSB immunoprecipitates was analyzed by northern blotting. Total RNA extracted from 10% of cell lysates and 5% of cell lysates (in the inputs) were also subjected to northern blotting (A) and immunoblotting (B) analyses, respectively. In (C), copy numbers of tRF_U3_1 and its precursor were determined as described in Supplementary Figure S2. The copy number ratios of tRF_U3_1 to pre-tRNA in the input and immunoprecipitated La are presented in (D). White asterisk in (B) indicates the La/SSB. Error bars are standard deviations of three independent experiments. *P values were calculated by unpaired Student t-test. **P < 0.01.
Figure S6. tRF_U3_1 is not functionally linked to the miRNA/RNAi pathway. (A) No interaction between tRF_U3_1 and Ago2 assessed by a pull-down assay followed by immunoblotting analysis. (B) Bioinformatic analysis of smRNAs associated with Ago2 immunoprecipitated from HeLa (GSE45506) and FT3-7, a Huh7-derived cell line (GSE57381). Scatter plot of miRNAs (left) or tRF_U3 family tRFs (right) comparing input and immunoprecipitated (IP) samples. X-axis represents the abundance (log10 transformed average RPM value) of the indicated smRNAs in the input sample. Y-axis represents their abundance (average RPM value) in Ago2-IP normalized to the indicated control IP. (C) RNAi activity of tRF_U3_1 was assessed in CCD-18Co cells transfected with a dual-luciferase reporter plasmid containing the complementary sequence of tRF_U3_1 at the 3′-UTR region of the Renilla luciferase gene. At 24 h post-transfection of the reporter and tRF_U3_1 or its derivatives (10 nM each), relative normalized luciferase activity (Rluc/Fluc) was determined. Duplex tRF_U3_1 is an siRNA mimic formed with tRF_U3_1 and its antisense form (AS_tRF_U3_1). n.s., not significant (unpaired two-tailed Student t-test). (D) Predicted binding sites at the 3′-UTR of the potential tRF_U3_1 targets CDK2AP2 and EIF3B. (E) RNAi activity of tRF_U3_1 was assessed as described in (C) using a luciferase reporter carrying the 3′-UTR of CDK2AP2 (psiCHECK-2_CDK2AP2-3′UTR) or EIF3B (psiCHECK-2_EIF3B-3′UTR). 

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mRNA levels of predicted target of tRF_U3_1 in Huh7 cells transfected with tRF_U3_1 (100 nM). GAPDH-normalized target mRNA levels are shown as fold-changes relative to the mock-treated control.

Immunoblotting analysis of tRF_U3_1-protein complexes pulled down from cell lysates revealed that tRF_U3_1, unlike miRNA and siRNA, did not form an RNA-induced silencing complex (RISC) by binding to Ago2. Rather, tRF_U3_1 was associated with the La/SSB (Supplementary Figure S6A). By bioinformatic analysis of Ago2-associated miRNAs and tRF_U3 family tRFs in HeLa and FT3-7 cells, we found that tRF_U3_1 read count in the Ago2-IP sample was below baseline values in both cell lines, whereas a substantial portion of miRNAs, irrespective their expression levels, were loaded onto Ago2 (Supplementary Figure S6B). Even the miRNAs whose expression levels were lower than that of tRF_U3_1 were enriched in the Ago2-IP samples compared to control IP samples, suggesting that tRF_U3_1 was not selectively bound to Ago2. Unlike tRF_U3_1, a limited number of tRF_U3 family tRFs were associated with Ago2 in both cell lines. Further studies are warranted to investigate whether these potentially Ago2-loadable tRFs are capable of regulating target gene expression.

Using a reporter plasmid carrying tRF_U3_1 target, we demonstrated that tRF_U3_1 and its isomer tRF_U3_1(3′-4U) indeed did not display RNAi, whereas a double-stranded tRF_U3_1 siRNA mimic (duplex tRF_U3_1), but not the AS_tRF_U3_1, dramatically reduced the reporter expression (Supplementary Figure S6C). We also investigated whether four selected potential cellular mRNA targets of tRF_U3_1 (which were among the top 64 targets with a ΔG value < −20 kcal/mol) could be negatively regulated by RNAi. Our analysis for two selected targets, cyclin-dependent kinase 2-associated protein 2 (CDK2AP2) and eukaryotic translation initiation factor 3B (EIF3B), revealed that neither of these targets, via use of the Renilla luciferase reporter system, was silenced by tRF_U3_1 (Supplementary Figure S6D and E). Furthermore, endogenous mRNA levels of these targets and the other two predicted targets, cell division cycle 6 (CDC6) and TNF receptor-associated factor 7 (TRAF7), were largely unaffected (Supplementary Figure S6F). These results further strengthen our conclusion that RNAi-mediated host mRNA expression regulation is unlikely to constitute the mode of action of tRF_U3_1.
Figure S7. Critical role of 3′-end U-tail in regulation of La/SSB-dependent HCV IRES-mediated translation. (A) HCV IRES-mediated translation was monitored in CCD-18Co cells transfected with the indicated single-stranded RNA oligonucleotides including tRF_U3_1 derivatives (0.1 nM). as_IRES, an antisense RNA targeting HCV IRES pseudoknot I (nt 317–337); tRF-5001, a tRF_5 family tRF [can be derived from tRNA-Ser(GCT) (chr6.tRNA61, chr15.tRNA10, chr17.tRNA123, chr11.tRNA8, chr6.tRNA43, and chr6.tRNA43)]. (B) Northern blot analysis of total RNA (15 µg) from two independent Huh7 cell cultures (#1 and #2) for tRF-5001, which was identified previously in other cell lines by Lee et al. (1). Synthetic tRF-5001 (0.1 ng) was loaded as a control for the assay. Error bars are standard deviations of at least three independent experiments. ***P < 0.001; n.s., not significant by unpaired two-tailed Student’s t-test.

The results showed that ssRNAs bearing no U-tail at their 3′-end (as_IRES and tRF-5001, a tRF_5 family tRF not detected in Huh7) had no significant effect on La/SSB-dependent HCV IRES-mediated translation. No inhibitory activity of tRF-5001 suggested that La/SSB function was not interfered by the 5′-end region tRF separated from mature tRNA. More importantly, tRF_U3_1(ΔU17-19) as well as MT(3′-CC) did not display the inhibitory activity. In contrast, tRF_U3_1 and its two tRF_U3_1 isomers bearing either 2U or 3U at their 3′-end significantly reduced the reporter activity, demonstrating further the important role of terminal U residues.
Figure S8. Antiviral activity of tRF_U3_1 in HCV subgenomic replicon-replicating cells. (A) HCV genome and protein levels were measured by RT-qPCR and immunoblotting, respectively, three days post-transfection of tRF_U3_1 into R-1 cells harboring an HCV subgenomic replicon (top). Transfection of single stranded miR-122-5p and its duplex form were used as assay controls. (B) Effect of a tRF_U3_1-targeting antisense oligonucleotide in R-1 cells. R-1 cells were transfected with anti-tRF_U3_1_PNA or other control PNAs (PNA_SL3-17 targets the HCV X-RNA stem-loop 3 at the 3′-end of the viral genome, and PNA-UpFS targets the −1 frame-shifting site of the SARS-CoV genome). After three days, HCV subgenomic RNA and NS5B protein levels were determined by RT-qPCR and immunoblotting. Error bars are standard deviations of three independent experiments. **P < 0.01; ***P < 0.001; by unpaired two-tailed Student’s t-test.
Figure S9. Validation of no expression of mature form tRNA-Ser(TGA) in tRF_U3_1 KO cell lines and RNAi-mediated depletion of tRF_U3_2 in Huh7 cells. (A) Northern blotting analyses of total RNA from Huh7 and tRF_U3_1 KO cell lines using the indicated probes. (B) tRF_U3_1 KO cell line #1 was transfected with an empty vector or ptRNA-Ser(TGA) plasmid. Two days after transfection, total RNA from transfected cells and Huh7 cells, along with the in vitro transcript (IVT, 101-nt) of pre-tRNA-Ser(TGA) (100 and 500 pg), was analyzed by northern blotting using the indicated probes. (C) Partial sequence of pre-tRNA-Val(TAC) (chr10.tRNA6) with its 3′-end trailer, tRF_U3_2. Target site of sitRF_U3_2 and its predicted cleavage sites are indicated. (D) Experimental scheme for the stem-loop RT-qPCR used for quantification of trailers (see the detailed in the Materials and Methods). (E) Depletion of tRF_U3_2 and its precursor was verified by a real-time RT-qPCR using a stem-loop primer (for reverse transcription) and a pair of specific forward and reverse primers. Intracellular tRF_U3_1 and tRF_U3_2 levels in Huh7 cells transfected with a control siRNA (siCtrl) or sitRF_U3_2 (50 nM) were determined at 2 days post-transfection. From the same RNA samples, smRNAs were recovered following electrophoresis on an 8 M-15% acrylamide gel and subjected to RT-qPCR to determine the tRF_U3_2 levels normalized to tRF_U3_1 levels. U6 snRNA was used as an internal control for quantification of trailers in total RNA. Error bars are standard deviations of three independent experiments with triplicate assays. ***$P < 0.001$; n.s., not significant by unpaired two-tailed Student’s $t$-test.
Figure S10. Impact of La/SSB knockout on Huh7 proliferation. (A) CRISPR-Cas9-mediated generation of La/SSB KO Huh7-derived cell lines. Loss of La/SSB expression in two independent La/SSB KO cell lines were verified by immunoblotting with two different anti-La/SSB monoclonal antibodies. (B) Growth kinetics of La/SSB KO cell lines. Error bars are standard deviations of at least three independent experiments. *P < 0.05; **P < 0.001; by unpaired two-tailed Student’s t-test.
Figure S11. Loss of La/SSB expression promotes destabilization of the trailer-derived tRFs. Northern blot analysis for the tRF_U3_2 (A) and tRF_U3_5 (B), which are the tRF_U3 family tRFs derived from pre-tRNA trailers, and their corresponding tRNA precursors in Huh7 cells and in the indicated two independent La/SSB knockout cell lines. EtBr, ethidium-bromide staining.
Figure S12. Effect of RNAi-mediated acute depletion of La/SSB on tRF_U3_1 stability. (A) Northern and immunoblot analyses of total RNAs and cell lysates, respectively, from La/SSB-depleted Huh7 cells following siLa (20 nM) transfection for the indicated time periods. Cellular abundances of tRF_U3_1 along with its precursor (top) and miR-122 (bottom) were analyzed by northern blotting. Of note, tRF_U3_1 decay appeared to be delayed in RNAi-mediated La-depleted cells, suggesting another layer of regulation induced at the early stage of La depletion. Further studies are required to address this possibility. (B) Decay kinetics of tRF_U3_1 in cellular extracts of tRF_U3_1 KO Huh7 cells transfected with siCtrl or siLa. Residual tRF_U3_1 levels at the indicated time points were quantified by Phosphorimager analysis, following northern blotting. Error bars are standard deviations of three independent experiments. $P$ values were calculated by unpaired Student $t$-test. *$P < 0.05$; **$P < 0.01$. 
Figure S13. Both tRF_U3_1 and its precursor binds to and stabilized by La/SSB. The Flag-tagged human La (Flag-hLa) was immunoprecipitated from cell lysates of tRF_U3_1 KO Huh7-derived cell line transfected with a plasmid expressing pre-tRNA-Ser(TGA) with or without pFlag-hLa. RNA in the immunoprecipitated Flag-hLa was analyzed by northern blotting as described with the indicated probes. Total RNA extracted from 10% of cell lysates and 5% of cell lysates (in the inputs) were also subjected to northern blotting (A) and immunoblotting (B) analyses, respectively. Shown in (C and D) are band intensities of pre-tRNA and trailer tRF (C) and their ratios (D) in the input and immunoprecipitated hLa. Error bars are standard deviations of three independent experiments. P values were calculated by unpaired Student t-test. **P < 0.01; ***P < 0.001.
Figure S14. Inhibition PV IRES-mediated translation by synthetic tRF_U3_1. Inhibitory effect of synthetic tRF_U3_1 on poliovirus IRES-mediated translation in La/SSB KO cells transiently expressing hLa. Error bars are standard deviations of three independent experiments. *P < 0.05; **P < 0.01.
Figure S15. Model for inhibition of La/SSB-dependent viral gene expression by the trailer tRFs. Pre-tRNA synthesized by RNA polymerase III undergoes processing to remove a short 5′-leader sequence and the 3′-trailer in the nucleus. It has long been believed that the trailers are degraded after being released by RNase Z (ELAC1/2)-mediated processing. Our results show that a distinct set of pre-tRNAs might be aberrantly exported from the nucleus by an as yet unknown mechanism and be processed irrespective of the presence or absence of La/SSB in the cytoplasm. Accumulation of the trailer-derived tRFs through stabilization by La/SSB-binding to the trailer 3′ uridylate residues renders cells resistant to the RNA viruses, which usurp La/SSB as a proviral host factor, by sequestering this RNA chaperone from viral cis-acting RNA elements such as HCV and poliovirus IRES and norovirus 3′-UTR. Yet, several important questions still remain unanswered, including how pre-tRNA is exported to the cytoplasm; whether pre-tRNA processing in the cytoplasm for a certain set of tRNAs represent a general event that has not yet been explored; the mechanism by which these trailer-derived tRFs in the cytoplasm and nucleus are decayed; and whether there is an active stabilization pathway for cytoplasmic accumulation of free trailer in liver cancer cells. As we found that La/SSB is not indispensable in pre-tRNA processing, it would also be of interest what other alternative proteins with RNA chaperone activity can replace the function of La/SSB in pre-tRNA processing in mammalian cells.
SUPPLEMENTARY TABLES

Table S1. List of probes used for northern blot analysis

Table S2. tRF_U3 family tRFs identified by deep-sequencing of small RNAs in Huh7 cells

Table S3. Small RNA datasets used in this study

Table S4. Complete list of tRF_U3 family tRFs and miRNAs differentially expressed in non-cancerous and cancerous human liver tissues

Table S5. Human and mouse tRNA-Ser(TGA)-coding genes

Table S6. Human tRNA-Ser-coding genes

Table S7. List of tRFs used in this study
| Target | Probe sequence (5′–3′) | Length (nt) | tRNA | Genome locus |
|--------|------------------------|-------------|------|--------------|
| tRF_U3_1 | AAATAAGAGCACCCGCTTC | 19 | Ser(TGA) | chr10.tRNA2 |
| tRF_U3_2 | AAACAACAGACCACACC | 17 | Val(TAC) | chr10.tRNA6 |
| tRF_U3_5 | AAAGTAAGCCACCACAT | 17 | Ser(GCT) | chr15.tRNA10 |
| tRF_U3_20 | AAACAGTAGCCACGA | 15 | Pro(TGG) | chr14.tRNA6 |
| trNAser(TGA) 5′-end region | ACCACTGCGAGATCGTGC | 19 | Ser(TGA) | chr10.tRNA2 |
| trNAser(TGA) 3′-end region (probe #1) | CGCACGCGGAGTTTCG | 18 | Ser(TGA) | chr10.tRNA2 |
| trNAser(TGA) 3′-end region (probe #2) | CAGCGAGCAGGTTTCGAAC | 19 | Ser(TGA) | chr10.tRNA2 |
| trNAser(TGA) 3′-end region (probe #3) | CGCAGCGGAGCGTTTCGA | 20 | Ser(TGA) | chr10.tRNA2 |
| RF-5001 | CCACCGGACACCTCGTC | 18 | Ser(GCT) | chr6.tRNA62 chr15.tRNA10 chr17.tRNA7 chr6.tRNA123 chr11.tRNA8 chr6.tRNA43 chr6.tRNA31 |
| snoU38b | AGAACTGACAAAGTTTCATCAG | 24 |
| miR-122 | ACAACACACCACAGACACTCAA | 23 |
| 5S rRNA | CCTGCTAGCTCAGTTGAGATCA | 21 |
| pre-miR-886 | AGGGTCAAGCAGACCCCGCG | 21 |
Table S2. tRF_U3 family tRFs identified by deep-sequencing of small RNAs in Huh7 cells

| tRF_U3 | RPM  | Major sequence (5′ to 3′) | tRNA | tRNA loci |
|--------|------|---------------------------|------|----------|
| tRF_U3_1 | 1761 | GAAGCGGGUUCUCUUAAUUU | SerTGA | chr10.tRNA2 |
| tRF_U3_2 | 628  | UGGUGUGUUCUUGUUGUUU | ValTAC | chr10.tRNA6 |
| tRF_U3_3 | 401  | AGGGGUGGCUUUGUUU | ThrCGT | chr6.tRNA1A21 |
| tRF_U3_4 | 339  | AUAGGUAUUAAGGCUUUU | AlaTGC | chr6.tRNA66 |
| tRF_U3_5 | 235  | AUGUGGUGGUCCUUACUUU | SerGCT | chr15.tRNA10 |
| tRF_U3_6 | 231  | UUCAAAGGUUACGGUUU | GlnTTG | chr6.tRNA64 |
| tRF_U3_7 | 195  | AACCGAGGGCUUACGGUUU | ThrAGT | chr19.tRNA4 |
| tRF_U3_8 | 192  | UAGCCGUUUCGCGGCUU | ValTAC | chrX.tRNA4 |
| tRF_U3_9 | 159  | UGACUUGACCUUCUUU | GluTTC | chr15.tRNA11 |
| tRF_U3_10 | 147 | GUAAUCUCUGUGCUUU | IleAAT | chr6.tRNA59 |
| tRF_U3_11 | 147 | AGGCAGUACGUAGAUUU | AlaCGC | chr6.tRNA119 |
| tRF_U3_12 | 136 | ACAAGUGCCGUUACUUU | TyrGTA | chr8.tRNA4 |
| tRF_U3_13 | 129 | GCCGUAGAUAACUUU | ThrTGT | chr14.tRNA20 |
| tRF_U3_14 | 125 | ACCUCAGAAGUCUCACCUU | LeuTAC | chr16.tRNA27 |
| tRF_U3_15 | 114 | CCUGUUGGCUCCUUUU | ThrTGT | chr15.tRNA56 |
| tRF_U3_16 | 61  | GCUAAGGAGUCCUCUGCUUGCUUUU | SerGCT | chr17.tRNA7 |
| tRF_U3_17 | 57  | UGUGUGUAGUACGUUUU | CysGCA | chr17.tRNA26 |
| tRF_U3_18 | 51  | GAGAAGCGUCUGCACUUU | PheGAA | chr6.tRNA96 |
| tRF_U3_19 | 43  | AGGGAGGCUUUAAGUAAACUUU | ArgTCG | chr15.tRNA4 |
| tRF_U3_20 | 39  | UCGUGUGUUCGCUUUU | ProTG | chr14.tRNA6 |
| tRF_U3_21 | 32  | GUAAAGCCGUUUAACGUUU | GluTCT | chr1.tRNA59 |
| tRF_U3_22 | 23  | UUGCCAUUGUAAACGUUU | ArgCCG | chr6.tRNA1 |
| tRF_U3_23 | 22  | GCACUGGUGCUUAAU | LysCTT | chr5.tRNA9 |

a tRF_U3 family tRFs were sorted in descending order of their RPM values.
b Average RPM values were obtained by analyzing two independent small RNA libraries of Huh7 cells (GSE128164), and all isoforms (≥ 10 reads) with different lengths of 3′-end U-tail were included in the analysis.
c Shown is the major isoform sequence detected in one of the small RNA libraries of Huh7 cells (Huh7-1: GSM3666020).
### Table S3. Small RNA datasets used in this study

| Library   | Sample used   | Description                                      | Tissue               | Age | Sex | Reference |
|-----------|---------------|--------------------------------------------------|----------------------|-----|-----|-----------|
| GSE57381  | GSM1381481    | Liver tissue from control subject                | Human liver          | 53  | M   | (2)       |
| GSE57381  | GSM1381482    | Liver tissue from control subject                | Human liver          | 34  | M   |           |
| GSE57381  | GSM1381483    | Liver tissue from control subject                | Human liver          | 78  | M   |           |
| GSE57381  | GSM1381484    | Liver tissue from control subject                | Human liver          | 75  | M   |           |
| GSE128164 | GSE128164     | Healthy volunteers (NL-1)                         | Normal liver tissue  | 43  | M   | This study|
| GSE128164 | GSE128164     | Healthy volunteers (NL-2)                         | Normal liver tissue  | 56  | M   |           |
| ERX015606 | ERX015606     | Human6 liver                                     | Human6 liver         | 36  | M   |           |
| ERX015607 | ERX015607     | Human7 liver                                     | Human7 liver         | 36  | M   |           |
| ERX015608 | ERX015608     | Human8 liver                                     | Human8 liver         | 36  | M   |           |
| ERX015609 | ERX015609     | Human9 liver                                     | Human9 liver         | 36  | M   |           |
| ERX015610 | ERX015610     | Human10 liver                                    | Human10 liver        | 36  | M   |           |
| GSE57381  | GSM1381489    | Chronic hepatitis B                              | HCC                  | 61  | M   | (2)       |
| GSE57381  | GSM1381490    | Chronic hepatitis B                              | HCC                  | 46  | M   |           |
| GSE57381  | GSM1381491    | Chronic hepatitis B                              | HCC                  | 59  | M   |           |
| GSE57381  | GSM1381492    | Chronic hepatitis B                              | HCC                  | 47  | M   |           |
| GSE57381  | GSM1381497    | Chronic hepatitis C                              | HCC                  | 68  | M   |           |
| GSE57381  | GSM1381498    | Chronic hepatitis C                              | HCC                  | 64  | M   |           |
| GSE57381  | GSM1381499    | Chronic hepatitis C                              | HCC                  | 59  | M   |           |
| GSE57381  | GSM1381500    | Chronic hepatitis C                              | HCC                  | 60  | M   |           |
| GSE57381  | GSM1381501    | Metastatic liver cancer                          | Liver cancer         | 70  | M   | (3)       |
| GSE57381  | GSM1381502    | Metastatic liver cancer                          | Liver cancer         | 70  | M   |           |
| GSE57381  | GSM1381503    | Metastatic liver cancer                          | Liver cancer         | 74  | M   |           |
| GSE57381  | GSM1381504    | Metastatic liver cancer                          | Liver cancer         | 44  | M   |           |
| GSE57381  | GSM1381505    | Metastatic liver cancer                          | Liver cancer         | 49  | F   |           |
| GSE57381  | GSM1381506    | Metastatic liver cancer                          | Liver cancer         | 66  | F   |           |
| GSE57381  | GSM1381507    | Metastatic liver cancer                          | Liver cancer         | 64  | F   |           |
| GSE57381  | GSM1381508    | Metastatic liver cancer                          | Liver cancer         | 64  | F   |           |
| GSE95683  | GSE95683      | SSBR PAR-CLIP, RNase A                            | HEK293               |     |     | (4)       |

*Huh7-derived stable cell line expressing TLR3. UK, unknown; HCC, hepatocellular carcinoma.
Table S4. Complete list of tRF_U3 family tRFs and miRNAs differentially expressed in non-cancerous and cancerous human liver tissues

|                  | Base Mean | log2 fold-change | P value  | P_adj    |
|------------------|-----------|------------------|----------|----------|
| tRF_U3_1         | 1578      | 5.32             | 1.13E-05 | 2.34E-04 |
| tRF_U3_17        | 134       | 2.57             | 5.19E-03 | 2.76E-02 |
| tRF_U3_3         | 89        | 6.24             | 1.24E-03 | 9.15E-03 |
| tRF_U3_11        | 53        | 9.25             | 3.57E-05 | 5.40E-04 |
| hsa-miR-21-5p    | 96643     | 1.48             | 7.23E-06 | 1.65E-04 |
| hsa-miR-29a-3p   | 15165     | 2.06             | 4.48E-06 | 1.20E-04 |
| hsa-miR-25-3p    | 14679     | 1.54             | 4.99E-07 | 1.90E-05 |
| hsa-let-7e-5p    | 7072      | 1.65             | 1.03E-02 | 4.79E-02 |
| hsa-miR-451a     | 5398      | -1.31            | 7.84E-04 | 6.51E-03 |
| hsa-miR-26b-5p   | 4961      | -1.12            | 2.82E-05 | 4.78E-04 |
| hsa-miR-28-3p    | 4473      | -1.06            | 1.90E-03 | 1.24E-02 |
| hsa-miR-221-3p   | 3803      | 3.47             | 1.54E-15 | 7.03E-13 |
| hsa-miR-27a-3p   | 3116      | 0.91             | 8.56E-05 | 1.06E-03 |
| hsa-miR-93-5p    | 2834      | 1.18             | 3.13E-03 | 1.81E-02 |
| hsa-miR-423-3p   | 2431      | 0.92             | 2.61E-04 | 2.77E-03 |
| hsa-miR-23a-3p   | 2254      | 1.94             | 1.11E-05 | 2.34E-04 |
| hsa-miR-130a-3p  | 2211      | -0.85            | 4.25E-03 | 2.34E-02 |
| hsa-miR-30e-5p   | 1293      | -1.18            | 1.80E-03 | 1.21E-02 |
| hsa-miR-148b-3p  | 1285      | -1.19            | 3.66E-05 | 5.40E-04 |
| hsa-miR-148a-5p  | 1222      | -1.46            | 6.36E-03 | 3.23E-02 |
| hsa-miR-203a     | 1216      | 4.22             | 4.69E-07 | 1.90E-05 |
| hsa-miR-215-5p   | 1017      | 3.63             | 2.17E-08 | 1.65E-06 |
| hsa-miR-200c-3p  | 1006      | 6.08             | 3.20E-09 | 5.80E-07 |
| hsa-miR-532-5p   | 995       | 1.20             | 1.90E-03 | 1.24E-02 |
| hsa-miR-425-5p   | 636       | 1.49             | 3.79E-05 | 5.41E-04 |
| hsa-miR-200b-3p  | 551       | 3.23             | 2.92E-05 | 4.78E-04 |
| hsa-miR-15b-5p   | 549       | 1.26             | 2.68E-05 | 4.78E-04 |
| hsa-miR-130b-3p  | 511       | 0.99             | 7.25E-04 | 6.25E-03 |
| hsa-miR-106b-5p  | 487       | 1.62             | 3.53E-07 | 1.61E-05 |
| hsa-miR-199b-5p  | 438       | 3.02             | 2.59E-05 | 4.78E-04 |
| hsa-miR-210-3p   | 432       | 1.38             | 5.33E-04 | 4.97E-03 |
| hsa-miR-183-5p   | 430       | 2.14             | 2.26E-03 | 1.42E-02 |
| hsa-miR-200b-5p  | 428       | 5.63             | 4.76E-06 | 1.21E-04 |
| hsa-miR-374a-5p  | 387       | 0.60             | 9.66E-03 | 4.60E-02 |
| hsa-miR-885-3p   | 342       | -5.40            | 2.74E-07 | 1.39E-05 |
| hsa-miR-1269a    | 330       | 5.36             | 2.62E-03 | 1.60E-02 |
| hsa-miR-17-3p    | 229       | 1.09             | 5.18E-03 | 2.76E-02 |
| hsa-miR-181d-5p  | 229       | 3.56             | 1.93E-08 | 1.65E-06 |
| hsa-miR-221-5p   | 227       | 3.41             | 9.12E-03 | 4.39E-02 |
| hsa-miR-196b-5p  | 218       | 8.00             | 1.73E-06 | 5.66E-05 |
| hsa-miR-421      | 211       | 1.49             | 5.98E-05 | 8.14E-04 |
| hsa-miR-139-5p   | 192       | -1.87            | 3.32E-05 | 5.23E-04 |
| miRNA              | log2FC | log10(padj) | log10(baseMean) | log10(FDR) |
|--------------------|--------|-------------|-----------------|-----------|
| hsa-miR-222-3p     | 2.57   | 3.81E-08    | 2.49E-06        |           |
| hsa-miR-500a-3p    | 1.97   | 3.81E-09    | 5.80E-07        |           |
| hsa-miR-1307-3p    | 1.78   | 1.04E-04    | 1.22E-03        |           |
| hsa-miR-548k       | 1.30   | 7.61E-03    | 3.78E-02        |           |
| hsa-miR-34c-5p     | 6.40   | 8.15E-07    | 2.87E-05        |           |
| hsa-miR-499a-5p    | 3.31   | 1.26E-03    | 9.15E-03        |           |
| hsa-miR-1304-3p    | 5.96   | 5.51E-03    | 2.86E-02        |           |
| hsa-miR-96-5p      | 3.95   | 1.45E-08    | 1.65E-06        |           |
| hsa-miR-30c-2-3p   | -1.51  | 2.23E-04    | 2.42E-03        |           |
| hsa-miR-92a-1-5p   | 1.97   | 1.10E-02    | 4.90E-02        |           |
| hsa-miR-378a-5p    | -1.70  | 2.59E-03    | 1.60E-02        |           |
| hsa-miR-125b-2-3p  | -1.91  | 1.37E-03    | 9.66E-03        |           |
| hsa-miR-25-5p      | 8.06   | 1.78E-07    | 1.01E-05        |           |
| hsa-miR-502-3p     | 1.91   | 1.04E-03    | 8.08E-03        |           |
| hsa-miR-135b-5p    | 6.52   | 7.79E-05    | 9.89E-04        |           |

Differential expression was analyzed using DESeq2. tRF_U3 family tRFs and miRNAs with a Padj < 0.05 and baseMean > 50 are listed. miRNAs are listed in descending order according to their baseMean values. It is of note that only tRF_U3_1 has a >1,000 baseMean among tRF_U3 family tRFs.
| Species | Genome locus* | Chromosome number | tRNA # | Start | End | Strand | Mature tRNA sequence (5′-3′)* | 3′-trailer sequence (5′-3′)* |
|---------|---------------|-------------------|--------|-------|-----|--------|-------------------------------|-------------------------------|
| Human   | chr02         | tRNA10            | 132140142 | 132140211 | +   | GAGAAGGTCATAGAGGTATGGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | ^CTTAGGTCTCTCATGTAGGTTGTTGCTCT CGAATGTCGATGATGGTTGTTGTTGAA... |
|         | chr02         | tRNA21            | 131033024 | 131033093 | -   | GAGAAGGTCATAGAGGTATGGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | ^CTTAGGTCTCTCATGTAGGTTGTTGCTCT CGAATGTCGATGATGGTTGTTGTTGAA... |
|         | chr06         | tRNA51            | 27513468  | 27513549  | +   | GTAGTCGTGGCAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | CTAGGGTCTCTCATGTAGGTTGTTGCTCT CTAGGGTTTT |
|         | chr06         | tRNA148           | 27473607  | 27473688  | -   | GTAGTCGTGGCAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | CTAGGGTCTCTCATGTAGGTTGTTGCTCT CTAGGGTTTT |
|         | chr06         | tRNA172           | 26312806  | 26312805  | +   | GTAGTCGTGGCAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | CTAGGGTCTCTCATGTAGGTTGTTGCTCT CTAGGGTTTT |
|         | chr10         | tRNA2             | 69524261  | 69524342  | +   | GCAGCGATGGCGAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | CTAGGGTCTCTCATGTAGGTTGTTGCTCT CTAGGGTTTT |
|         | chr17         | tRNA32            | 22028179  | 22028247  | -   | GAAAAGTCATAGGGTTAGGAGCGCTCTGCTGAAACCAGGCGAATGTCGATGATGGTTGTTGTTGAA... | CTAGGGGTTTT |
| Mouse   | chr10         | tRNA1105          | 63429557  | 63429476  | -   | GCAGCGATGGCGAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | GAAGCGGGGTTCTCCTTATTTT |
|         | chr13         | tRNA112           | 23518146  | 23518227  | +   | GTAGTCGTGGCAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | TGAATGATTTTT |
|         | chr13         | tRNA996           | 21925016  | 21924935  | -   | GTAGTCGTGGCAGTGGAATTGGATTGGCTTGAAACCAGTGCTCTGCGGTTGAGTTCCCTCCTTTTCCA | TGAATGATTTTT |

*a All tRNA-Ser(TGA) sequences in this table were obtained from the Genomic tRNA Database (http://gtrnadb.ucsc.edu).
*b **“CCA”**, the unique sequence post-transcriptionally added to the 3′-end of mature tRNA, is not shown.
*c Shown are the trailer sequences, located downstream of the mature tRNA 3′-end, which bear the first four “T” residues in the predicted pre-tRNA transcripts.
*d For the trailer sequences that lack the four “T” residues within the 50 bases downstream of the mature tRNA 3′-end, the first 50 nt sequences in the genome are shown.
| Species | Anticodon | Chromosome number | tRNA # | Start | End | Strand | Mature tRNA sequence (5′-3′) | Predicted 3′-trailer sequence (5′-3′) |
|---------|------------|------------------|--------|-------|------|--------|----------------------------|-------------------------------------|
| Human   | AGA        | chr06            | tRNA5  | 26327817 | 26327898 | +     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA44 | 27446591 | 27446672 | +     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA66 | 27463593 | 27463674 | +     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA47 | 27470818 | 27470899 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA50 | 27499987 | 27500068 | +     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA115| 27521192 | 27521273 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA117| 27509554 | 27509635 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr06            | tRNA117| 27509554 | 27509635 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTAGGTGTTT                                      |
|         |            | chr07            | tRNA32 | 14930546 | 14930558 | +     | GCGTTGAATGCGTACGGGCTAAGTTTGTGCCACAGGGGCTGCTGCCAGGATCCTGCGAACGAGTTT | TCAATTATT                                      |
|         |            | chr08            | tRNA11 | 96281885 | 96281966 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGAATGTCAGAGTTT                                     |
|         |            | chr11            | tRNA64 | 10327098 | 10327063 | +     | CAAAGAAATTCATAGGAGGATACGTAGGCTAACGAGTTTAGGTGATAAGGGGGTGCTGCCAGAACGAGTTT | CTAAGGTGTTT                                      |
|         |            | chr11            | tRNA65 | 8129925 | 8130099 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | TCAATTATT                                      |
|         |            | Chr08            | tRNA35 | 27177590 | 27177709 | +     | GCTGTGATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | CTTCCTGATACCTTCCTCCCTTTT                                      |
|         |            | Chr06            | tRNA137| 27640229 | 27640310 | +     | GCTGTGATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | TGGCCTATTATT                                      |
|         |            | Chr12            | tRNA2  | 56584148 | 56584229 | +     | GTCACGAGGTCGAGGCTGAGGATGCTGCCATACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | GGTATT                                      |
|         |            | Chr17            | tRNA41 | 8042199 | 8042280 | -     | GTCACGAGGTCGAGGCTGAGGATGCTGCCATACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | TAAACCTTATCATAGGTTGGAATCCTGCAAGGCGG          |
|         |            | Chr06            | tRNA31 | 27065085 | 27065166 | +     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr06            | tRNA43 | 27265775 | 27265856 | +     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr06            | tRNA62 | 28180815 | 28180896 | +     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr06            | tRNA123| 28565117 | 28565198 | +     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr06            | tRNA175| 26305721 | 26305798 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr11            | tRNA8  | 66155931 | 6615672 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr15            | tRNA10 | 40880203 | 40880210 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr17            | tRNA8  | 8000104 | 8000106 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | ACGCTTATT                                      |
|         |            | Chr02            | tRNA68 | 15210148 | 15210211 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | CTTAGGTGCTTACATCTACGGTTGCTACGTGAGTTGAGTTGGCCAGG          |
|         |            | Chr02            | tRNA69 | 15103302 | 15103309 | -     | GAGCAGAATGCGGCTGCTGCTGCTGCTTGACAGGGCTACCAATGGGCTTCGCCAGCAGGTGAAATCCTGCAAGGCGG          | CTTAGGTGCTTACATCTACGGTTGCTACGTGAGTTGAGTTGGCCAGG          |
|         |            | Chr06            | tRNA51 | 27513466 | 27513549 | +     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTATT                                      |
|         |            | Chr06            | tRNA148| 27473607 | 27473688 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTATT                                      |
|         |            | Chr06            | tRNA172 | 26312824 | 26312905 | -     | GAGTGTGCTGCAGGTTTAGGTGATAAGGGGGTGCTGCCAGGTGGAATCCTGCGAACGAGTTT   | GGTATT                                      |
|         |            | Chr10            | tRNA2  | 49524261 | 49524342 | +     | GACGCGAGGAGGAGGCTGCTGCTGCTGCTTGACAGGGAGGCTACCAATGGGCTTCGCCAGCAGGCTGAGGGTAGTTGAGTTGGCCAGG          | GAGCCGAGGAGGAGGCTGCTGCTGCTGCTTGACAGGGAGGCTACCAATGGGCTTCGCCAGCAGGCTGAGGGTAGTTGAGTTGGCCAGG          |
| Chr | TSS | TES | Seq  | Description |
|-----|-----|-----|------|-------------|
| chr17 | 22028179 | 22028247 | GAAAAAGCTACAGGGGCTTGAGGCTGGCTTGAAACCAGCCTtAGGAGGTTCAATTCCTTCCTTTTTTG | chr17 tRNA32 |
| chr02 | 121267680 | 121267735 | GGCTCATGCTCAAGGCTACGACTGGGACATCTGGAGGcCCTGGGGCCC | chr02 tRNA22 |
| chr06 | 27261671 | 27261744 | GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCTGCTACTAATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT | chr06 tRNA41 |

*a* All tRNA-Ser sequences in this table were obtained from the Genomic tRNA Database (http://gtrnadb.ucsc.edu).

*b* “-CCA”, the unique sequence post-transcriptionally added to the 3′-end of mature tRNA, is not shown. The sequences matching the “consensus” tRNA model used in Cove analysis are presented in uppercase letters, while introns and other nucleotides in non-conserved positions are denoted by lowercase letters.

*c* Shown are the trailer sequences, located downstream of the mature tRNA 3′-end, which bear the first four “T” residues in the predicted pre-tRNA transcripts.

*d* For the trailer sequences that lack the four “T” residues within the 50 bases downstream of mature 3′-end, the first 50-nt sequences in the genome are shown. Among a total of 32 tRNA-Ser species, seven species have the “TGA” anticodon. The four species in green represent nuclear-encoded mitochondrial tRNAs. Shown in yellow (3 species total) are pseudo-tRNA species. Per our analysis options (see Methods in the Supplementary Information), among the 32 predicted trailer sequences, only three species presented in sky blue were identified by deep sequencing of the small RNA library from Huh7 cells.
### Table S7. List of tRFs used in this study

| Name | Alternative name/ or isoform | Sequence (5′–3′) | Length (nt) | Genome locusa | Mapping on Pre-tRNA | Reference |
|------|-----------------------------|-----------------|-------------|--------------|---------------------|-----------|
| tRF_U3_1 | tRF-1001 | GAAGCGGGUGCUUAAUUU | 19 | chr10.tRNA2-Ser(TGA) | 3′-trailer | (1) |
| tRF_U3_1(3′-4U) | Cand45 | GAAGCGGGUGCUUAAUUU | 20 | chr10.tRNA2-Ser(TGA) | 3′-trailer | (6) |
| tRF_U3_1(3′-2U) | Eym65 | GAAGCGGGUGCUUAAUUU | 18 | chr10.tRNA2-Ser(TGA) | 3′-trailer | (7) |
| tRF_U3_1(ΔU17-19) | | GAAGCGGGUGCUUUA | 16 | chr10.tRNA2-Ser(TGA) | 3′-trailer | |
| tRF_U3_1_MT(3′-CC) | | GAAGCGGGUGCUUAAUCC | 19 | chr10.tRNA2-Ser(TGA) | 3′-trailer | |
| AS_tRF_U3_1 | AAAAAAGACACCACCUUU | | 19 | chr10.tRNA2-Ser(TGA) | 3′-trailer | |
| tRF_U3_2 | tRF-1010 | UGGUGUGUUGCUUGUUU | 18 | chr10.tRNA6-Val(TAC) | 3′-trailer | (1) |
| tRF_U3_5 | | AUGUGUGGCUUACUUUU | 18 | chr15.tRNA10-Ser(GCT) | 3′-trailer | |
| tRF_U3_16 | | GCUAAGGAAGUCCUGUCCAGUUUU | 26 | chr17.tRNA7-Ser(GCT) | 3′-trailer | (1) |
| tRF-1003 | | GCUAAGGAAGUCCUGUCCAGUUUU | 25 | chr17.tRNA7-Ser(GCT) | 3′-trailer | (1) |
| tRF_U3_20 | | UCGUGCUUCAGUUUUU | 17 | chr14.tRNA6-Pro(TGG) | 3′-trailer | (1) |
| tRF-1019 | | UCGUGGCUACAGUUUUU | 16 | chr10.tRNA2-Ser(TGA) | 3′-trailer | (1) |
| tRF-3002 | | ACCCUGCUCGUGCCCA | 18 | chr6.tRNA62-Ser(GCT) | chr15.tRNA10-Ser(GCT) | chr17.tRNA7-Ser(GCT) | chr16.tRNA123-Ser(GCT) | chr11.tRNA8-Ser(GCT) | chr6.tRNA43-Ser(GCT) | chr6.tRNA31-Ser(GCT) | 3′-end of mature tRNA | (1) |
| tRF-5001 | | GACGAGGGUGCCAGUGG | 18 | chr6.tRNA62-Ser(GCT) | chr15.tRNA10-Ser(GCT) | chr17.tRNA7-Ser(GCT) | chr16.tRNA123-Ser(GCT) | chr11.tRNA8-Ser(GCT) | chr6.tRNA43-Ser(GCT) | chr6.tRNA31-Ser(GCT) | 5′-end of mature tRNA | (1) |

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a The tRNA number on a chromosome is based on the tRNA# in the Genomic tRNA Database (http://gtrnadb.ucsc.edu/).
Supplementary Materials and Methods

Plasmids, antibodies, and reagents

The bicistronic vector pDual-IRES, which expresses a cap-dependent Renilla luciferase (Rluc) reporter and an HCV IRES-mediated firefly luciferase (Fluc) reporter, was described previously (8). The bicistronic vector pRPF (9), which expresses Fluc via PV IRES, was received from Dr. Sung Key Jang (POSTECH, Pohang, Korea). The pJFH1 plasmid (10), which was used to produce infectious HCV (genotype 2a) particles, was provided by Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan). To overexpress the La/SSB protein, the pFlag-hLa plasmid was constructed by inserting the La/SSB cDNA (RefSeq Accession NM_001294145), amplified by reverse transcription (RT)-PCR using forward (5′-AAGCTTGGTGCTGAAAATGGTGATAATGAAAAG-3′) and reverse (5′-GGATCCCTACTGGTCTCCAGCACC-3′) primers, into HindIII and BamHI sites of the pFLAG-CMV-4 vector (Sigma-Aldrich, St. Louis, MO, USA). The pFlag-hLaΔNLS plasmid, which expresses human La/SSB with a deletion of amino acids 375–408 including the NLS (11), was generated by PCR using pFlag-hLa as a template. The dual luciferase reporter psiCHECK-2_tRF_U3_1, containing the tRF_U3_1 complementary sequence at the 3′-UTR of the Rluc reporter, was constructed by inserting XhoI and NotI-digested duplex DNA, formed with the tRF_U3_1 forward primer (5′-TATCCCCTC4AGAAATAAGAGACACCGCTTCGC4GGCCCGC4CGGTAAA-3′) and the tRF_U3_1 reverse primer (5′-TTACCGGCGGCCGCGGATA-AAATAAGAGCACCCGCTTCGCGGCCGCCGTAAA-3′) carrying NotI (underlined) and XhoI (italic and underlined) sites, in the psiCHECK-2 vector (Promega, Madison, WI, USA), which was digested with the same enzymes. Similarly, the dual luciferase reporters containing the 3′-UTR cDNA of CDK2AP2 (RefSeq Accession NM_005851.3: nt 931–1378) and EIF3B (RefSeq Accession NM_001037283.1: nt 2529–3081) were constructed by conventional RT-PCR followed by inserting the PCR products into psiCHECK-2 to generate psiCHECK-2_CD42AP2_3′UTR and psiCHECK-2_EIF3B_3′UTR, respectively. The ptRNA-Ser(TGA) vector expressing tRNA-Ser(TGA) on chromosome 10 was PCR-amplified from Huh7 genomic DNA, using the forward primer 5′-CCTGGCATGAGCGCAGTGGTTGTTACACTA-3′ and the reverse primer 5′-TGCTCTTCTCATGGGAGCAAGAGTAGTTTACACTA-3′ and the reverse primer 5′-TGTCCTTCTCATGGGAGCAAGAGTAGTTTACACTA-3′. The 381-bp DNA fragment was cloned into the pCR2.1-Topo vector (Invitrogen, Carlsbad, CA, USA). The IRF3-5D plasmid expressing a constitutively active IRF3 and the IFNβ-pGL3 luciferase reporter expressing firefly luciferase under the control of the IFN-β promoter (16) were kindly provided by John Hiscott (McGill University, Montreal, Quebec, Canada) and used for the interferon β reporter assays. Polynosinic:polycytidylic acid ([poly(I:C)]), a double-stranded RNA analog, was obtained from Sigma-Aldrich. HCV 3′-untranslated region (UTR) in vitro transcript was prepared by T7 RNA polymerase-mediated in vitro transcription using T7 MEGAscript kit (Ambion, Austin, TX, USA) and a template amplified from pZS2 plasmid (17) (kindly provided by Dr. Christoph Seeger, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA, USA), an HCV subgenomic replicon derived from the parental HCV Con-1 replicon 1377/NS3′ (AJ242652), by PCR using a set of primers [forward primer, 5′-TTAATACTAGACTCATATAGGGTGAGGAGCATCTCTC-3′ (T7 promoter: underlined) and reverse primer, 5′-ACATGATCTGAGAGAGGCC-3′] (18). Antibodies were obtained as follows: mouse anti-α-tubulin antibody (clone DM1A; 1:5,000 dilution) from Calbiochem (La Jolla, CA, USA), rabbit polyclonal anti-β-actin antibody (EB967; 1:5,000 dilution) and rabbit polyclonal anti-Ago2 antibody (C34C6; 1:1,000 dilution) from Cell Signaling (Beverly, MA, USA), mouse monoclonal anti-Lamin A/C antibody (clone 14/LaminAC; 1:1,000 dilution) from BD Bioscience (Franklin Lakes, NJ,
USA), mouse monoclonal anti-La/SSB antibodies (clones 312B and 44; 1:1,000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal anti-Flag antibody (#F7425, 1:1,000 dilution) from Sigma-Aldrich, and rabbit polyclonal anti-norovirus capsid protein VP1 (ab92976, 1:1,000 dilution) from Abcam (Cambridge, UK). Recombinant La/SSB protein expressed in E. coli was obtained from GenWay Biotech, Inc. (GWB-T00740; San Diego, CA, USA).

**Synthetic small RNAs, antisense oligonucleotides, and transfection**

All chemically synthesized small RNAs were purchased from ST Pharm (Seoul, Korea) or Bioneer (Daejeon, Korea). All siRNAs had a UU-3′ overhang and their sequences are as follows: sitRF_U3_2 [siRNA targeting the 3′-trailer region of pre-tRNA-Val(TAC), 5′-UGGAACCAUGGGUGUUUU-3′ (passenger stand) and 5′-AGACCACACCAUGGUUCAUU-3′ (guide strand)]; siLa [siRNA targeting La/SSB (19), 5′-ACAACAGACUUUAAUGUAUU-3′ (passenger strand) and 5′-UUACAUUAAAGUCUGUGUUUU-3′ (guide strand)]; and siCtrl [control siRNA targeting GFP, 5′-CUCGCGGCACACGCUGAACU-3′ (passenger stand) and 5′-GUUCAGCGUGUCCGGCAU-3′ (guide strand)]. as_IRES (5′-CAGGGUCUACGAGCCUCGCC-3′), an antisense RNA targeting HCV IRES pseudoknot I spanning nt 317−337 was used as a control. All synthetic small RNAs had 5′ monophosphate and 3′ OH unless otherwise specified. miR-122 duplex consisted of 5′-AACGCCAUUAUCACACUAAAUA-3′ (passenger strand) and 5′-UGGAGUGUGACAAUGGUGUUU-3′ (guide strand, miR-122-5p). Antisense peptide nucleic acids (PNAs) were purchased from Panagene Inc. (Daejeon, Korea): Anti-tRF_U3_1, N-AAATAAGACCCCGCTTC-C; SL3-17, N-CGTAAGATGGAGCCACC-C; and Up-PS, N-GGGTTCGCGGAGTTG-C.

siRNA (20 nM unless otherwise indicated), miRNAs (20 or 100 nM) and tRFs (0.1, 10, or 100 nM), were transfected into cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions, as indicated in the figure legends. Transfection into primary cells was assisted using the ND98 lipidoid (98N12-5) as a transfection reagent that was kindly provided by Professor Seung-Woo Cho (Yonsei University, Seoul, Korea). RNA-lipidoid complexes were formulated as described previously (20). Briefly, lipidoid, which was diluted in 25 mM sodium acetate buffer (pH 5.0) at a final concentration of 0.5 mg/ml, and RNA were mixed with a weight ratio of 5(lipidoid):1(RNA). The mixture was then incubated for 20 min at room temperature to allow complex formation. The resulting lipidoid/RNA complexes were added to Opti-MEM (Gibco-BRL, Gaithersburg, MD, USA; for RAW264.7) or complete Dulbecco’s modified Eagle’s medium (DMEM) [Lonza, Walkersville, MD, USA; for human peripheral blood mononuclear cells (hPBMCs)] and dropwise applied to cells. The sequences of tRFs used in this study are provided in Supplementary Table S7.

For PNA transfection, cells (3 × 10^6) in 400 µl Opti-MEM (Gibco-BRL, MD, USA) were electroporated with each PNA at 260 V and 950 µF in a 4 mm electrode gap cuvette (Bio-Rad, CA, USA) using a Gene Pulser II electroporator (Bio-Rad). After electroporation, cells were incubated at room temperature for 5 min, resuspended in pre-warmed complete DMEM, and then split into one 10-cm dish or three 6-cm dishes. Cells were harvested three days after electroporation for analysis of HCV genome and protein levels by quantitative RT-PCR and western blot analysis, respectively.

**Cell culture**

The human hepatocellular carcinoma cell line Huh7 and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Lonza), 2 mM L-glutamine, 100 U/ml of penicillin, 100 µg/ml streptomycin and 0.1 mM nonessential amino acids at 37°C in
5% CO₂. The Huh7-derived cell line R-1, which supports stable, autonomous replication of genotype 1b HCV subgenomic replicon RNA, was cultivated as described previously (21). The murine macrophage cell line RAW264.7, human embryonic kidney 293 (HEK293) or 293T (HEK293T), and human colon fibroblast CCD-18Co cells were also similarly cultivated in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin. Huh7, HeLa (CCL-2), HEK293 (CRL-1573), HEK293T (CRL-3216), CCD-18Co (CRL-1459), and RAW264.7 (TIB-71) cells were similarly cultivated in DMEM supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin.

Establishment of tRF_U3_1 and La/SSB knockout cell lines

The tRF_U3_1 knockout Huh7 cells were generated using the CRISPR-Cas9 genome editing system as described previously (22). Briefly, a 20-bp guide sequence (5′-ACCCGCTTCCGACGCGAGCA-3′) targeting the mature tRNA-Ser(TGA) gene on chromosome 10 was selected from the candidate sites predicted by Off-Spotter (23). Two complementary oligos 5′-CACCACCCGCTTCCGACGCGAGCA-3′ and 5′-AAACTGCTCGCTGCGGAAGCGGGTC-3′, containing the guide sequence were annealed and phosphorylated. The annealed oligo was then ligated into the BbsI-digested pX330 vector (Addgene, Cambridge, MA, USA). Similarly, La/SSB KO Huh7 cells were generated using the two 20-bp guide sequences (5′-TTGGCGACTTCAATTTGCCA-3′ and 5′-TCCTTTAGAACTTGTCCCG-3′) targeting the La/SSB-coding gene on chromosome 2, which were selected from the GeCKO v2 library (24) and cloned into the pX330 by the procedure described above. Huh7 cells grown in 24-well culture plates were transfected with 500 ng of the vector using FuGENE HD transfection reagent (Promega). At 72 h post-transfection, serially diluted cells were plated in 10-cm dishes to isolate single cell-derived colonies. Candidate KO colonies were screened by sequencing and verified by northern blotting or immunoblotting. For sequencing, genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega), and the target loci were PCR-amplified and cloned into the pCR2.1-Topo vector (Invitrogen) for sequencing analysis.

Virus infection and transfection

Infectious JFH1 HCV RNA (genotype 2a, AB047639) was prepared by in vitro transcription using the T7 MEGAscript kit (Ambion) and electroporated into Huh7 cells as described previously (21). Cell culture-derived HCV (HCVcc) was collected from culture media by centrifugation at 2,000 × g for 10 min, followed by passing through a 0.45-μm filter. Huh7 cells were infected with HCVcc by adsorption for 12 h. Cells were then washed with PBS and further cultivated in complete DMEM. For transient RNA replication, JFH1 RNA prepared by in vitro transcription was transfected into human primary hepatocytes using the TransIT-mRNA transfection kit (Mirus Bio LLC, Madison, WI, USA). Murine norovirus 1 (MNV-1) strain CW1 (kindly provided by Prof. Herbert Virgin IV, Washington University School of Medicine, St. Louis, MO, USA) was propagated in RAW264.7 cells (25). Plaque forming assay for MNV-1 was performed as described previously (26).

Small RNA library construction

Total RNA was extracted from Huh7 cells and human liver tissues with TRIzol reagent (Invitrogen) and purified according to manufacturer’s recommendations. The isolated RNA
was prepared for Illumina sequencing using the TruSeq Small RNA Sample Preparation kit (Illumina) according to manufacturer’s protocol. Briefly, RNA was ligated with 3′ (5′-ppTGGGAATTCTCGGGTGCCAAGGNH2-3′) and 5′ (5′-GUUCAGAGUUCACAGUCCGACGAUC-3′) adapters using T4 RNA ligase and was reverse-transcribed to cDNA with the Illumina RNA RT primer (5′-GCCTTGGCACCAGGAATTTCA-3′). The cDNA was amplified using Illumina RNA PCR primer sets (5′-GCCTTGGCACCAGGAATTTCA-3′ and 5′-CAAGCAGAAGACGGCATACGAGATTCCTTGGCACCCGAGAATTCCA-3′). The PCR products were size-fractionated by electrophoresis on a 5% Tris/borate/EDTA polyacrylamide gel to recover DNA fragments in the range of 140–180 bp by electroelution. The resulting small RNA library was limited to small RNAs with a 5′ monophosphate because adapter ligation requires this terminal moiety.

Mapping and annotation of small RNAs
The reads (≥ 10 reads) were mapped using the Bowtie (version 1.0.1) software (27) with the “-v 2 -m 10 --best --strata” options. Reads that could be aligned to the human genome were moved to the “mapped” data set and suppressed reads were discarded to prevent multiple mapping for further alignment. The remaining reads were aligned to the tRNA sequences in a custom tRNA database, in which mature tRNA contained the post-transcriptionally-added CCA at the 3′ end of mature tRNA sequences (28), allowing identification of tRF_3 family tRFs. After mapping, only unique reads of the “mapped” data set were annotated using GtRNAdb (http://gtrnadb.ucsc.edu (29)): for tRNA-derived tRFs matching to mature tRNA sequences without any mismatches), miRDB (miRbase database release v20, http://www.mirbase.org) (30), and the database for known non-coding RNAs (ensembl database Homo_sapiens.GRCh37.75.ncrna.fa, http://www.ensembl.org). The reads annotated as ribosomal RNAs were removed. For read analysis for tRNA-derived tRFs, the sequences matching to both tRNA sequences and other genome sequences were excluded. For sequences matching to more than one tRNA, read number was divided equally into individual tRNAs.

Bioinformatic analysis of small RNA deep sequencing datasets
The small RNA high throughput sequencing data for human non-cancer liver tissue (n = 22), liver cancer tissue (n = 14), and HCC lines (n = 5) were collected from in-house small RNA libraries or downloaded from the NCBI Gene Expression Omnibus (GEO) and the Sequence Read Archive (SRA). The accession numbers of datasets from NCBI GEO and SRA and details of the in-house libraries are presented in Supplementary Table S3. Small non-coding RNA profiling for these datasets was performed using the procedure described above. The DESeq2 package (31) was used for differential expression analysis for the tRF_U3 family and miRNAs with standard parameters. The log2 fold-changes of tRF_U3 family and miRNA reads (Padj < 0.05 and baseMean ≥ 50) from the non-cancerous and cancerous liver tissues are listed in Supplementary Table S4. The La PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation) assay datasets (4) were downloaded from the GEO database (GSE95683). Reads (≥ 16 nt) from the PAR-CLIP datasets were aligned using the Bowtie software (27) to the tRF_U3 family tRFs with the allowance of two mismatches.

Nano LC-MS/MS analysis
Nano LC-MS/MS analysis was performed on an Agilent 1100 Series nano-LC and LTQ-mass spectrometer (Thermo Electron, San Jose, CA, USA) using a capillary column (150 mm × 0.075 mm; Proxeon, Odense M, Denmark) packed in house with 5 μm, 100-Å-pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA, USA). Mass spectra were
acquired using data-dependent acquisition with full mass scan (350–1800 m/z) followed by MS/MS scans. The mascot algorithm (Matrix Science, Boston, MA, USA) was used to identify peptide sequences present in a protein sequence database.

Subcellular fractionation
Huh7 cells suspended in buffer A [250 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail (Roche, Basel, Switzerland)] were lysed by passing through a 30-G needle 10 times using a 1-ml syringe. After 20 min incubation on ice, unbroken cells and nuclei were pelleted by centrifugation at 720 × g for 5 min. The cytosolic fraction was obtained from the supernatant collected by centrifugation at 100,000 × g for 1 h. Unbroken cells and nuclei pellets were washed with buffer A and passed through a 30-G needle 10 times. The nuclei pellet collected by centrifugation at 1,000 × g for 10 min was then suspended in buffer B [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, and protease inhibitor cocktail (Roche)] and sonicated on ice two times for 3 sec, prior to analysis by northern- and immuno-blottings.

Northern blot analysis
For small RNA detection, DNA oligonucleotide probes were end-labeled with [γ-32P] ATP using T4 polynucleotide kinase (Takara, Kyoto, Japan). Total RNA (20 µg) extracted from cells using TRIzol reagent (Invitrogen) was resolved by electrophoresis on a 15% polyacrylamide-8 M urea gel and electrically transferred to positively charged nylon membranes (Roche). The membrane was UV-crosslinked, pre-hybridized with salmon sperm DNA (20 µg/ml; Invitrogen), and then probed in hybridization buffer (5× SSPE, 5× Denhardt’s solution, and 0.5% SDS). After washing twice at 37°C with a washing buffer solution (6× SSC and 0.5% SDS) for 5–15 min, the blot was analyzed by Phosphorimager (BAS-2500; Fuji Film, Tokyo, Japan).

Real-time RT-qPCR
Total RNA was extracted with TRIzol (Invitrogen) and purified according to the manufacturer’s procedures. Intracellular HCV RNA levels were quantified using the iQ Supermix RT-PCR kit (Bio-Rad) as described previously (32). MNV RNA levels were quantified as described previously (33). Fluorescence was detected using the CHROMO4 continuous fluorescence detector (MJ Research, Waltham, MA, USA). RT-qPCR analysis of mRNA levels was performed using SYBR Premix ExTaq (Takara) and specific oligonucleotide primers for target sequences. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the endogenous control. The following primers were used as described previously (34) and elsewhere: IFN-β, forward primer 5′-GAACCTTGACATCCCTGAGGAGATTAAGCAGC-3′ and reverse primer 5′-GTTCCTAGGATTTCCACTCTGATGGTCC-3′; CDK2AP2, forward primer 5′-TGTTTAACGACTTTGGACCGC-3′ and reverse primer 5′-CCATCTCCTATGACTGACCAGC-3′; CD6, forward primer 5′-ACCTATGCAACACTCCCCATT-3′ and reverse primer 5′-TGGCTAGTTCTCTTTTGCTAGGA-3′; TRAF7, forward primer 5′-TCTGCGCTCCACATTCTCAC-3′ and reverse primer 5′-ACCGCGATGTCTTTCAACC-3′; U6 snRNA, forward primer 5′-CTCGCTTCGGGAGCGACATATACT-3′ and reverse primer 5′-AAGGTCTGGAGAGGTCGGATGC-3′ and reverse primer 5′-GAAGATGGTGATGGGATTTC-3′. For U6 snRNA cDNA synthesis, an RT-primer 5′-
AAAATATGGAACGCTTCACGAATTTG-3’ was used. Target gene levels normalized to GAPDH or U6 snRNA were determined using the \( \Delta \Delta C_t \) method (35). miRNA levels were quantified by TaqMan probe-based real-time PCR assays (Applied Biosystems, Foster, CA, USA) as described previously (36). Expression of pre-tRNA trailers were quantified by stem-loop RT-qPCR according to previously published protocols (37,38) with slight modifications. Briefly, reverse transcription was carried out in a total volume of 15 \( \mu l \) of reaction mixture containing total RNA (1 \( \mu g \)) or gel-eluted small RNA and a stem-loop primer (5’-CTCAACTGGTGGACAGTGCGCAATTCAGTTGAGAAATAGA-3’ for tRF_U3_1 and 5’-CTCAACTGGTGGACAGTGCGCAATTCAGTTGAGAAACCAAC-3’ for tRF_U3_2) by incubation for 30 min at 16°C and for 30 min 42°C. After further incubation at 85°C for 5 min, the reaction mixture (1.3 \( \mu l \)) was used for PCR using SYBR premix ExTaq (Takara) and a pair of specific oligonucleotide primers (250 nM each). The universal reverse primer sequence is 5’-ACACTCCAGCTGGGGAAGCGGGTGCTCTTTA-3’. The forward primer sequences are 5’-ACACTCCAGCTGGGGAAGCGGGTGCTCTTTA-3’ for tRF_U3_1 and 5’-ACACTCCAGCTGGGGAAGCGGGTGCTCTTTA-3’ for tRF_U3_2. The PCR started with an initial incubation at 95°C for 12 min, followed by 35 cycles of 15 s at 95°C and 1 min at 60°C.

**Immunoblotting and immunostaining analyses**

Cells were lysed in a lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100] supplemented with an EDTA-free protease inhibitor cocktail (Roche) by incubating on ice for 20 min. Cleared cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose Hybond ECL membrane (GE Healthcare, Uppsala, Sweden) in a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked with 5% BSA in TBST buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20] and then probed with primary antibodies. Bound antibody was detected using appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoblots were developed using the ECL detection kit (GE Healthcare Life Sciences, Piscataway, NJ, USA). For immunostaining of La/SSB, La/SSB KO Huh7 cells grown on Lab-Tek 4-well chamber slides (Nunc, Roskilde, Denmark) were transfected with pFlag-hLa or pFlag-hLa(ΔNLS) and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min. After washing 3 times with PBS, cells were permeabilized by incubating with 0.1% Triton X-100 in PBS for 10 min, washed 3 times with PBS, and blocked by incubating with 1% BSA in PBS for 1 h at room temperature. Cells were then further incubated with a monoclonal anti-La/SSB antibody for 2 h. After washing 3 times with PBS, appropriated fluorescent-conjugated secondary antibodies were used to visualize the antigen. After staining nuclei with 1 \( \mu M \) 4’,6’-diamidino-2-phenylindole (DAPI) for 10 min, fluorescent signal was visualized using a confocal laser scanning microscope (Zeiss LSM 880, Carl Zeiss, Oberkochen, Germany).

**Pull-down experiments**

Cell lysates were prepared using a lysis buffer [50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.5% Igepal CA-630, 0.5 mM DTT, and protease inhibitor cocktail tablets (1 tablet per 10 ml; Roche), and RNAsin RNase inhibitor (4 U/ml, Promega)]. After removing cell debris by centrifugation, supernatants were mixed with 1 \( \times \) 10\(^{13}\) copies of 5’-biotinyl tRF_U3_1 (5’-biotin-C\( _{18} \) linker-GAACCGGUGCUCUUAAUUU-3’; obtained from Bioneer). After incubation for 2 h at 4°C on an orbital rotator, pre-washed Dynabeads M-280 Streptavidin (approximately 500-\( \mu g \) beads; Invitrogen) were added into the reaction mixture. After 30 min incubation, the beads were washed five times with 1-ml of fresh lysis buffer. The pulled-down proteins were subjected to immunoblotting or LC-MS/MS analysis.
RNA immunoprecipitation
Huh7 or tRF_U3_1 KO cell line (clone #2) grown on a 15-cm plate were lysed by suspending in 1.5 ml of lysis buffer [25 mM Tris–HCl (pH 7.4), 150 mM KCl, 2 mM EDTA, 1 mM NaF, 1% NP40, and protease inhibitor cocktail (Roche)] on ice for 30 min. After removing nuclei and unbroken cells by centrifugation at 17,000 × g for 30 min, cleared cell lysates (ca. ~1.4 ml) were harvested and used for La immunoprecipitation, RNA preparation (for Northern blotting), and immunoblotting analyses using 85%, 10%, and 5% of the lysates, respectively. Endogenous La/SSB protein was immunoprecipitated from cell lysates supplemented with RNase inhibitor (100U/ml, Promega) using a La/SSB-specific monoclonal antibody [clone 312B (1 μg); Santa Cruz Biotechnology], which was prebound to 30 μl of Dynabeads Protein G (30 mg/ml; Invitrogen) by incubating in the above described lysis buffer (1 ml). Normal mouse IgG (1 μg; Merck Millipore, Burlington, MA, USA) was used as a control. For immunoprecipitation of Flag-tagged hLa from tRF_U3_1 KO cells, anti-FLAG M2 agarose beads (30 μl of a 50% slurry, Sigma Aldrich) was used. After 4 h incubation with rotation at 4°C, the beads were washed four times with the lysis buffer (1.2 ml each) and resuspended in the same buffer (500 μl). The resulting immunoprecipitates (90%) were treated with proteinase K (40 μg) for 30 min at 50°C in 200 μl of reaction buffer [200 mM Tris–HCl (pH 7.5), 300 mM NaCl, 10 mM EDTA, and 0.5% SDS]. RNA was then recovered by acidic phenol-chloroform extraction. Total RNA (20 μg) from tRF_U3_1 KO Huh7 cells was added to the RNA extracted from immunoprecipitated samples to enhance recovery of small RNAs by ethanol/3M sodium acetate precipitation. The remaining beads (10%) were used for immunoblotting analysis.

Electrophoretic mobility shift assay
HCV IRES RNA was internally labeled with [α-32P]-UTP by in vitro transcription using PCR-amplified HCV IRES cDNA and the T7 MEGAscript kit (Ambion) according to the manufacturer’s instructions. The cDNA template containing HCV IRES region (nt 1–361, genotype 1b HCV) was amplified from the pZS2 plasmid using Ex-Taq polymerase (Takara) and forward (5′-TAATACGACTCACTATAGGGCGGCCAGCCCAGATGGGGGGCG-3′; T7 promoter sequence is underlined) and reverse (5′-GGTTAGGATTCGTGCTCATGG-3′) primers. In vitro transcript RNA was resolved by electrophoresis on a 5% polyacrylamide/8 M urea gel and eluted from the gel with elution buffer [0.5 M ammonium acetate, 10 mM magnesium acetate tetrahydrate, 1 mM EDTA (pH 8.0), and 0.1% SDS]. RNA was then recovered by acidic phenol-chloroform extraction. tRF_U3_1 probe was prepared by 5′-end radiolabeling using [γ-32P]-ATP and T4 polynucleotide kinase. Radiolabeled RNA probes were incubated with La/SSB protein with or without unlabeled competitors in a total volume of 10-μl binding buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 5 mM MgCl2, 10% glycerol, and RNasin (1 U/μl)] at 4°C for 15 min followed by further incubation at room temperature for 30 min. After incubation, 2-μl non-denaturing loading buffer (50% glycerol, 0.01% xylene cyanol, and 0.01% bromophenol blue) was added, and reaction mixtures were subjected to electrophoresis on a 5% non-denaturing polyacrylamide gel.

Cell viability assay
Cytotoxicity of tRF_U3_1 was measured using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent (Promega) according to the manufacturer's protocol. Briefly, R-1 cells grown in a 96-well plate (2 × 104 cells per well) were transfected with tRF_U3_1. Two days later, MTS substrate was added to each well. After 1 h incubation at 37°C, absorbance at 450 nm was read on a 96-well microplate reader (GloMax-Multi Detection system, Promega).
Luciferase assay
Cells were transfected with a dual-luciferase reporter plasmid using FuGene HD transfection reagent (Promega). After 6 h, trailer-derived tRF or miRNA was transfected into cells using Lipofectamine RNAiMAX (Invitrogen). Renilla luciferase (Rluc) and firefly luciferase (Fluc) activities were measured 18 h later using the Dual-Glo luciferase assay system (Promega).

PAR-CLIP assay
PAR-CLIP assay was performed as described previously (39). Briefly, R-1 cells (40 x 15-cm plate) grown in medium supplemented with 100 µM 4SU for 14 h were irradiated with 365-nm ultraviolet light. After treating cell lysates with RNase T1 (Thermo Fisher, Waltham, MA, USA), La/SSB protein was immunoprecipitated with a La/SSB-specific monoclonal antibody bound to magnetic Protein G Dynabeads (Invitrogen). After RNase T1 digestion followed by dephosphorylation and radiolabeling of the RNA crosslinked to La/SSB, the protein-RNA complexes were subjected to SDS-PAGE and visualized by autoradiography. The complexes eluted from the gel using the D-Tube Dialyzer kit (molecular weight cut-offs of 3.5-kDa, Merck) were treated with proteinase K (Roche) and RNA was then recovered by acidic phenol-chloroform extraction and ethanol precipitation for RT-qPCR analysis.

IFN-β reporter assay and enzyme-linked immunosorbent assay
IFN-β promoter activity assays were performed in Huh7 cells using the IFNβ-pGL3 plasmid that expresses IFN-β promoter-controlled Fluc as described previously (20). Briefly, cells were transfected with the IFNβ-pGL3 plasmid along with the pRL-TK plasmid used for normalization of transfection efficiency. The reporter-transfected cells were transfected with tRF_U3_1 to measure Fluc/Rluc activity 24 h later. IFN-α secretion from hPBMCs was measured by enzyme-linked immunosorbent assay (ELISA) as described previously (40). Briefly, hPBMCs were isolated from healthy volunteers’ blood (obtained from the Blood Services, Western Blood Center of Korean Red Cross) by centrifugation over Ficoll-Hypaque (GE healthcare). Purified hPBMCs (1 x 10⁶ per well) were seeded in a 96-well plate and transfected with RNA using the ND98 lipidoid. After stimulation for 16 h, culture supernatants were collected for ELISAs using a human IFN-α-ELISA kit (ebioscience, San Diego, CA, USA).

In vitro tRF_U3_1 decay assay
Synthetic tRF_U3_1 (10 pmol) was incubated at 37°C with tRF_U3_1 KO Huh7 cell lysate (40 µg) in a 110-µl reaction buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% NP-40, and 10% glycerol] supplemented with complete EDTA-free 1× protease inhibitor cocktail (Roche Diagnostics) for 0, 30, 60, and 120 min. RNA extracted from a 25-µl aliquot of the reaction mixture was used for northern blot analysis.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Prism Software Inc., La Jolla, CA, USA). Results are presented as the means ± standard deviation (SD) from at least three independent experiments. The P-value was calculated using the unpaired Student's t-test. P < 0.05 was considered statistically significant.

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