Supporting Information

# DNA tiling enables precise acylation-based labeling and control of mRNA

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**DOI: 10.1002/anie.2021XXXXX**

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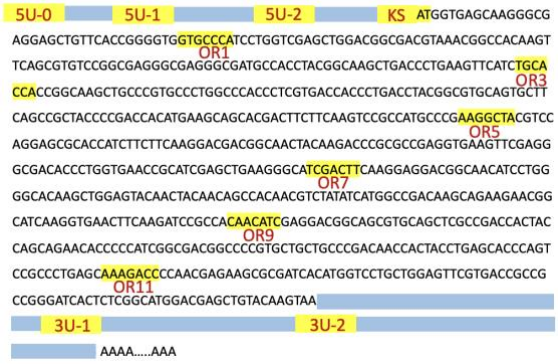
## Experimental Procedures

**Materials and instruments:** Reagents were purchased from Sigma-Aldrich unless specified otherwise. GFP mRNA and FLuc mRNA were purchased from TriLink (#L7201 and #L7202). 2M NAI-$N_3$ solution in DMSO was prepared according to the previously published procedures[1]. Cy5-DBCO and TAMRA-DBCO were purchased from Click Chemistry Tools. Wheat-germ *in vitro* translation kit, RNasin and DNase I were purchased from Promega. The SuperScript™ II Reverse Transcriptase, RNaseOUT™ Recombinant Ribonuclease Inhibitor and NTP Set (100 mM Solution), Messenger Max Lipofectamine, NuPAGE™ MES SDS Running Buffer (20X), NuPAGE™ Transfer Buffer (20X) were purchased from Thermo Fisher Scientific. PABP first antibody, G3BP1 first antibody and Alex405 anti-mouse secondary antibody were purchased from Cell Signaling Technology. IRD680 anti-mouse secondary antibody and IRD800 anti-rabbit secondary antibody were purchased from Li-Cor. Antarctic Thermolabile UDG, LunaScript® RT SuperMix Kit and Luna® Universal qPCR Master Mix were purchased from New England Biolabs (NEB). RNA clean-up and concentrator-5 column was purchased from Zymo. Luciferase assay kit was purchased from Promega. All DNA sequences were purchased from IDT.
Oligonucleotide concentrations were measured with a NanoDrop One microvolume UV-Vis spectrophotometer. Fluorescence of GFP from *in vitro* translation was measured with a Fluoroskan Ascent Microplate Fluorometer (ThermoFisher Scientific) or with a plate reader (Tecan). RT-qPCR was recorded by a StepOnePlus Real-Time PCR System (Applied Biosystems). Fluorescence of gel bands was recorded using a Typhoon 9500 laser scanner (GE Healthcare) at $\lambda_{ex}$ = 488 nm and $\lambda_{em}$ = 520 nm for SYBR Gold, and $\lambda_{ex}$ = 546 nm and $\lambda_{em}$ = 580 nm for TAMARA. PAGE gel images were analyzed and quantified with ImageJ software. Western blots were imaged on a Li-Cor instrument at $\lambda_{ex}$ = 700 nm or 800 nm. Cell imaging was performed on an Inverted Zeiss LSM 780 multiphoton laser scanning confocal microscope. All quantitative experiments were performed in triplicate (except where noted), and the results averaged.

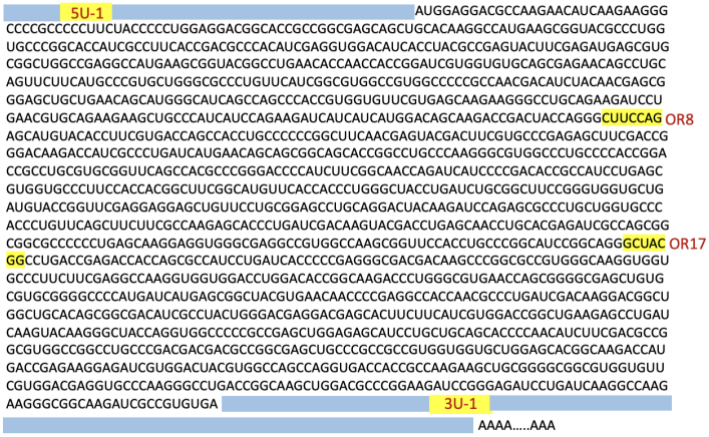
**General procedures for site-selective acylation of mRNA via TRAIL method:** 500 ng (~1.5 pmole) of mRNA and 1 ug (~3 pmole each) of pre-mixed protector DNAs and inducer DNA were heated in folding buffer containing 50 mM NaCl to 65℃ for 5 min and chilled on ice to form an mRNA-DNA duplex with a specific loop structure via DNA tiling (For FLuc mRNA, mRNA and DNAs mix were annealed by heating at 95℃ for 1 min and cooling down to 25℃ at the of 6℃/min ). To the hybridized solutions were added 3.3 µL 3.3xMOPs buffer (333 mM MOPs, 20 mM $MgCl_2$, 333 mM NaCl, pH 7.5) and then 2 µL 0.25M NAI-$N_3$ stock in dry DMSO was added to the final concentration of 50 mM NAI-$N_3$. The mixture was incubated at 37℃ for 4 h and then 6 µL DNase I stock (10 µL DNase I for FLuc mRNA) and 4 µL $H_2O$ were added to digest the tiling DNAs at 37℃ for 1h. 1 µL 250 mM EDTA was added prior to the inactivation of DNase I at 65℃ for 5 min. After reaction, the mRNA was purified by Zymo RNA clean-up and concentrator-5 column following the manufacturer's standard long RNA purification procedure. mRNA concentration was determined with a Nanodrop One microvolume UV-VIS spectrophotometer. mRNA samples were stored at -80 °C.

GFP and FLuc mRNA sequence with all induced loop sites tested (highlighted in yellow) in this work:

GFP mRNA sequence:




FLuc mRNA sequence:




5'UTR and 3'UTR sequences (blue) are proprietary and can be obtained by directly contacting TriLink.

**General procedures for short RNA model test.** 100 pmole) of short RNA and 200 pmole inducer DNA were heated in folding buffer containing 50 mM NaCl to 65℃ for 5 min and chilled on ice to form an RNA-DNA duplex with a specific loop structure. To the hybridized solutions were added 3.3 µL 3.3xMOPs buffer (333 mM MOPs, 20 mM $MgCl_2$, 333 mM NaCl, pH 7.5) and then 2 µL 0.25M NAI-$N_3$ stock in dry DMSO was added to the final concentration of 50 mM NAI-$N_3$. The mixture was incubated at 37℃ for 4 h and then 2 µL DNase I was added to digest the DNAs at 37℃ for 1 h. 1 µL 250 mM EDTA was added prior to the inactivation of DNase I at 65℃ for 5 min. After reaction, the RNA was purified by precipitation and proceeded for mass spectrometry by MALDI-TOF and for gel analysis.

**General procedures of click reaction with acylated mRNA**

1 µg Acylated mRNA was reacted with 25 µM fluorophore-DBCO (Cy5/TAMRA) or Biotin-DBCO in $H_2O$ (or in 1xPBS). The reaction was incubated for 3 h at 37℃ and was purified by RNA clean-up column followed the standard protocol. The purified mRNA concentration was determined with a Nanodrop One microvolume UV-VIS spectrophotometer and the labeled mRNAs were stored at -80 °C.

**General procedures for *in vitro* translation of mRNA:** The procedure of mRNA *in vitro* translation followed a commercial protocol using a wheat germ extract translation system (Promega). Briefly, 12.5 µL wheat germ extract, 0.5 µL 40U/µL RNasin, 2 µL 1 mM complete amino acid mixture and 2 µL 1 mM KOAc were added as a premix solution. 100 ng mRNA was added to the premix solution in a microplate and incubated for 3 h at 25℃. The GFP mRNA translation efficiency was quantified by GFP fluorescence measured by Fluoroskan Ascent Microplate Fluorometer (ThermoFisher Scientific) at at $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 532 nm. The FLuc mRNA translation efficiency was measured by luciferase assay kit from Promega followed with manufacturer's protocol and the luminescence signal was acquired by Fluoroskan Ascent Microplate Fluorometer.

**General procedures for deacylation of acylated mRNA by phosphine treatment:** For *in vitro* activation, 3 mM TPPMS or THPP were added to the acylated mRNA in $H_2O$ and incubated at 37℃ for 3 h, followed by RNA clean-up column purification. For in situ activation, 3 mM THPP was directly added to wheat germ extract translation system along with acylated mRNA. For cellular activation, TPPMS or THPP were added to medium at indicated concentration (1 mM, 3 mM, 5 mM) and incubated at 37℃ for 1 h before changing the medium.

**Quantitative reverse transcriptase PCR (RT-qPCR) of mRNA:** 50 ng mRNA was first reverse transcribed with a LunaScript® RT SuperMix Kit (NEB). mRNA was added with 4 µL 5x RT SuperMix and $H_2O$ to a 20 µL total volume. The mixture was reacted at 25℃ for 2 min, 55℃ for 10 min, and 95℃ for 1 min. The resulting cDNA mixture was diluted 50,000-fold and 1 µL of the diluted cDNA was taken as template and added to 19 µL master mix for qPCR. The prepared master mix included 0.5 µL 1U/ µL Antarctic Thermolabile UDG, 10 µL Luna® Universal qPCR Master Mix, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer and 7.5 µL $H_2O$. The reaction was performed with the following program: 25℃ for 10 min, 95℃ for 1 min, (95℃ for 15 s, 60℃ for 30 s) *40 cycles. The Ct value was recorded on a real-time PCR instrument and the effective RNA level was calculated as 2^- ($Ct_{mRNA}$ – $Ct_{UT}$).

**Polyacrylamide gel electrophoretic analysis of reverse transcriptase (RT) stops:** 500 ng (~1.5 pmol) mRNA was mixed with 2 pmol RT Primer and 0.25 µL dNTP mix (10 mM each, Invitrogen), and incubated for 5 min at 65℃, then immediately chilled on ice for 2 min. Then 2 µL 5x First-Strand Buffer (Invitrogen), 1 µL 0.1 M DTT, 0.5 µL RNaseOUT and 0.25 µL Super Script II (200 U/µl, Invitrogen) were added to the final volume of 10 µL. The reaction was incubated with the following program: 25℃ for 10 min, 42℃ for 50 min, and 52℃ for 50 min. After the reaction, 10 µL loading dye (8 M Urea, 0.05% Orange G, 0.05% Bromophenol blue) was added and the mixture was denatured at 96℃ for 3 min and loaded on a denaturing 20% polyacrylamide gel (PAGE). Products were separated in a PAGE gel in 1x TBE (pH 8.3, Sigma Aldrich), 20 mA, ~2 h. The cDNA gel was visualized by fluorescence imaging (Typhoon, GE Healthcare).

**Western blot procedure**: 40 µL of prepared protein samples were loaded onto an SDS-PAGE gel for electrophoresis in 1xSDS running buffer, 158 V, ~30 min. Then the protein samples were transferred to a PVDF membrane in 1x transfer buffer, 150 mA, ~1h. After transblotting, the membrane section of interest was cut out and transferred to a covered container. The membrane was blocked with 10 mL 5% dry milk powder in 1xTBST buffer (Blocking buffer, containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature on a shaker. The blocking buffer was discarded and then 5 mL of primary antibody solution was added (diluting 2.5 µL antibody stock into 5 mL of blocking buffer) to cover the membrane and incubated on a shaker at 4℃ overnight. At the 2nd day, the antibody solution was discarded, and the membrane was washed with 10 mL 1xTBST buffer three times (10 min incubation per wash on the shaker). 10 mL of secondary antibody solution (diluting 1 µL secondary antibody stock in 10 mL of blocking buffer) was then added, and the membrane was incubated on the shaker for another 1 h at room temperature. After incubation, the membrane was washed again as above and was blot-dried with a large filter paper. The membrane was finally imaged with a Li-Cor imager.

**Ribosome stalling study of acylated-mRNAs:** 1 µg of mRNA (acylated ORF-acylated mRNA or untreated mRNA) was added with 12.5 µL wheat germ extract, 0.5 µL 40U/ µL RNasin, 2 µL 1 mM complete amino acid mixture and 2 µL 1 mM KOAc in a total volume of 25 µL for *in vitro* translation at 25℃ for 1.5 h. After incubation, 5 µL 3.2 M KOAc and 2 µL 16 mM puromycin were added to the mixture and the reaction was incubated at 25℃ for another 1 h. Then 11 µL of 4xSDS buffer was added to stop the reaction. The resulting samples were heated at 95℃ for 5 min and loaded onto 4%-12% SDS PAGE gels for western blotting (procedure as above).

**Pull-down assay by selectively biotin-labeled mRNA:** Pull-down assays were conducted according to the previously published procedure[2]. Briefly, for *in vitro* pull-down, HeLa cell lysate was first prepared as described with a final protein concentration of 6 µg/µL in PEB buffer (20 mM Tris-HCl pH7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.5% IGPAL CA630). Pulldown of mRNA-binding proteins was performed with 125 µL 6 µg/µL cell lysate, 1 µg of biotin-labeled mRNA, 50 µL 10 x protease inhibitor, 5 µL 40U/ µL RiboLock and 250 µL 2 x TENT buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl, 1% Triton X-100) in 500 µL final volume. The reaction was mixed by pipetting and incubated at room temperature for 30 min. 50 µL streptavidin coupled Dynabeads for each reaction were washed with 1 x TENT buffer using the magnetic stand. After incubation, 50 µL washed beads were added to the

mRNA-protein mixture and mixed by pipetting up and down. The reaction was incubated at room temperature for another 30 min with intermittent mixing by tapping the tube every ~5 min. The reaction tube was placed in the magnetic stand for 1 min to allow the beads to settle to one side of the tube and the supernatant was then discarded. The beads were washed with 1 mL ice cold 1 x TENT buffer 4 times. 40 µL of 1 x SDS buffer was then added to the magnetic beads and the sample was heated at 95℃ for 5 min. The supernatant samples were carried on to western blotting. For in cell pull-down experiments, 1 µg biotin-labeled mRNA was first transfected into HeLa cells by Lipofectamine MessengerMax followed the manufacturer's protocol. After 16 h incubation, cells were lysed and the pull-down procedure was performed as above.

**Cellular studies of selectively fluorophore-labeled mRNA:** All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37℃ in a humidified atmosphere containing 5% $CO_2$.

For general mRNA imaging, cells were plated into 35 mm-10 mm glass-bottom Petri dishes (WPI) in growth media and grown to 70-90% confluence before transfection. Cy5-labeled mRNAs were transfected using Lipofectamine MessengerMax (Invitrogen) following manufacturer's protocols with minor modifications. On the day of experiments, growth medium was removed, and cells were washed with PBS three times and the medium was replaced with 100 µL DMEM containing 10% FBS containing 0.9 µL Lipofectamine and 300 ng mRNA in 10 µL of Opti-MEM, and 1 µg/ml of Hoechst 33342. The cells were incubated for 4 h post-transfection, followed by washing with PBS and fixing with 4% formaldehyde for 30 min at room temperature. The cells were washed with PBS, then mounted onto a confocal microscope. A 25× oil immersion objective lens was employed for the imaging. A 405 nm laser was used to acquire the images of Hoechst 33342 with a 415–475 nm emission filter set. A 488 nm laser was used to acquire the images of eGFP with 500–615 nm emission filter set. A 633 nm laser was used to acquire the images of Cy5-labeled mRNAs with 645–715 nm emission filter set. Images were analyzed and processed with Image-J software.

For stress granule experiments, transfection procedures were the same as above except the amount of mRNA was reduced to 37.5 ng to achieve better resolution. To test the translation and localization of mRNA under stress conditions, 200 µM sodium arsenite was added to the cells at 37℃ for 30 min either before mRNA transfection for 3 h co-incubation or after 4 h post-transfection. The cells were then fixed with 4% formaldehyde for 10 min and washed with PBS three times. The cells then were permeabilized with 0.5% Triton-X in 1× PBS for 10 min at room temperature. After washing with PBS, the cells were incubated in a blocking solution that contains 3% BSA in 1× PBS for an hour at room temperature. The blocking solution was washed with PBS, then the cells were incubated with primary antibody (G3BP1 E9G1M, rabbit mAb, Cell Signalling Technology) diluted in antibody dilution buffer (1% BSA, 0.3% Triton-X in 1× PBS, 1:100 ratio) for 24 h at 4ºC. After the cells were washed with PBS three times, the cells were incubated with secondary antibody (Dnk pAb to Rb IgG, Alexa 405, abcam) diluted in antibody dilution buffer (1:500 ratio) for 1 h at room temperature. The cells were washed with PBS another three times, then mounted onto a confocal microscope. A 25× oil immersion objective lens was employed for the imaging. A 405 nm laser was used to acquire the images of stress granules (G3BP1) with a 415–475 nm emission filter set. A 488 nm laser was used to acquire the images of eGFP with 500–615 nm emission filter set. A 633 nm laser was used to acquire the images of Cy5-labeled mRNAs with 645–715 nm emission filter set. Images were analyzed and processed with Image-J software.

**Table S1.** List of mRNA and DNAs

| Name | Sequence (left to right: 5' to 3') |
|---|---|
| GFP mRNA (ORF sequence) | |
| | AUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGGUGCCCAUCCUGGUCGAGCUGGACGGCGACGUAAACGGCCACAAGUUCAGCGU<br>GUCCGGCGAGGGCGAGGGCGAUGCCACCUACGGCAAGCUGACCCUGAAGUUCAUCUGCACCACCGGCAAGCUGCCCGUGCCCUGGCCCA<br>CCCUCGUGACCACCCUGACCUACGGCGUGCAGUGCUUCAGCCGCUACCCCGACCACAUGAAGCAGCACGACUUCUUCAAGUCCGCCAUGC<br>CCGAAGGCUACGUCCAGGAGCGCACCAUCUUCUUCAAGGACGACGGCAACUACAAGACCCGCGCCGAGGUGAAGUUCGAGGGCGACACCC<br>UGGUGAACCGCAUCGAGCUGAAGGGCAUCGACUUCAAGGAGGACGGCAACAUCCUGGGGCACAAGCUGGAGUACAACUACAACAGCCACA<br>ACGUCUAUAUCAUGGCCGACAAGCAGAAGAACGGCAUCAAGGUGAACUUCAAGAUCCGCCACAACAUCGAGGACGGCAGCGUGCAGCUCG<br>CCGACCACUACCAGCAGAACACCCCCAUCGGCGACGGCCCCGUGCUGCUGCCCGACAACCACUACCUGAGCACCCAGUCCGCCCUGAGCA<br>AAGACCCCAACGAGAAGCGCGAUCACAUGGUCCUGCUGGAGUUCGUGACCGCCGCCGGGAUCACUCUCGGCAUGGACGAGCUGUACAAG<br>UAA |
| Firefly Luciferase (FLuc) mRNA (ORF sequence) | |
| | AUGGAGGACGCCAAGAACAUCAAGAAGGGCCCCGCCCCCUUCUACCCCCUGGAGGACGGCACCGCCGGCGAGCAGCUGCACAAGGCCAUG<br>AAGCGGUACGCCCUGGUGCCCGGCACCAUCGCCUUCACCGACGCCCACAUCGAGGUGGACAUCACCUACGCCGAGUACUUCGAGAUGAGC<br>GUGCGGCUGGCCGAGGCCAUGAAGCGGUACGGCCUGAACACCAACCACCGGAUCGUGGUGUGCAGCGAGAACAGCCUGCAGUUCUUCAU<br>GCCCGUGCUGGGCGCCCUGUUCAUCGGCGUGGCCGUGGCCCCCGCCAACGACAUCUACAACGAGCGGGAGCUGCUGAACAGCAUGGGCA<br>UCAGCCAGCCCACCGUGGUGUUCGUGAGCAAGAAGGGCCUGCAGAAGAUCCUGAACGUGCAGAAGAAGCUGCCCAUCAUCCAGAAGAUCA<br>UCAUCAUGGACAGCAAGACCGACUACCAGGGCUUCCAGAGCAUGUACACCUUCGUGACCAGCCACCUGCCCCCCGGCUUCAACGAGUACG<br>ACUUCGUGCCCGAGAGCUUCGACCGGGACAAGACCAUCGCCCUGAUCAUGAACAGCAGCGGCAGCACCGGCCUGCCCAAGGGCGUGGCC<br>CUGCCCCACCGGACCGCCUGCGUGCGGUUCAGCCACGCCCGGGACCCCAUCUUCGGCAACCAGAUCAUCCCCGACACCGCCAUCCUGAG<br>CGUGGUGCCCUUCCACCACGGCUUCGGCAUGUUCACCACCCUGGGCUACCUGAUCUGCGGCUUCCGGGUGGUGCUGAUGUACCGGUUCG<br>AGGAGGAGCUGUUCCUGCGGAGCCUGCAGGACUACAAGAUCCAGAGCGCCCUGCUGGUGCCCACCCUGUUCAGCUUCUUCGCCAAGAGC<br>ACCCUGAUCGACAAGUACGACCUGAGCAACCUGCACGAGAUCGCCAGCGGCGGCGCCCCCCUGAGCAAGGAGGUGGGCGAGGCCGUGGC<br>CAAGCGGUUCCACCUGCCCGGCAUCCGGCAGGGCUACGGCCUGACCGAGACCACCAGCGCCAUCCUGAUCACCCCCGAGGGCGACGACA |

AGCCCGGCGCCGUGGGCAAGGUGGUGCCCUUCUUCGAGGCCAAGGUGGUGGACCUGGACACCGGCAAGACCCUGGGCGUGAACCAGCG
GGGCGAGCUGUGCGUGCGGGGCCCCAUGAUCAUGAGCGGCUACGUGAACAACCCCGAGGCCACCAACGCCCUGAUCGACAAGGACGGCU
GGCUGCACAGCGGCGACAUCGCCUACUGGGACGAGGACGAGCACUUCUUCAUCGUGGACCGGCUGAAGAGCCUGAUCAAGUACAAGGGC
UACCAGGUGGCCCCCGCCGAGCUGGAGAGCAUCCUGCUGCAGCACCCCAACAUCUUCGACGCCGGCGUGGCCGGCCUGCCCGACGACGA
CGCCGGCGAGCUGCCCGCCGCCGUGGUGGUGCUGGAGCACGGCAAGACCAUGACCGAGAAGGAGAUCGUGGACUACGUGGCCAGCCAGG
UGACCACCGCCAAGAAGCUGCGGGGCGGCGUGGUGUUCGUGGACGAGGUGCCCAAGGGCCUGACCGGCAAGCUGGACGCCCGGAAGAUC
CGGGAGAUCCUGAUCAAGGCCAAGAAGGGCGGCAAGAUCGCCGUGUGA

| Protector DNAs (ORF region of GFP mRNA) | |
|---|---|
| OR-comp1 | GTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCAT |
| OR-comp2 | GTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCC |
| OR-comp3 | GGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCC |
| OR-comp4 | CTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAG |
| OR-comp5 | GAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTG |
| OR-comp6 | CAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAA |
| OR-comp7 | GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCAC |
| OR-comp8 | GTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT |
| OR-comp9 | GGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCC |
| OR-comp10 | GTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTC |
| OR-comp11 | GACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTA |
| OR-comp12 | TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAG |

| Protector DNAs (ORF region of FLuc mRNA) | |
|---|---|
| FL-OR0 | GGGGCCCTTCTTGATGTTCTTGGCGTCCTCCATNNNNNNNNNNNNNNNNNNNN |
| FL-OR1 | CTTCATGGCCTTGTGCAGCTGCTCGCCGGCGGTGCCGTCCTCCAGGGGGTAGAAGGGGGC |
| FL-OR2 | GATGTCCACCTCGATGTGGGCGTCGGTGAAGGCGATGGTGCCGGGCACCAGGGCGTACCG |
| FL-OR3 | GCCGTACCGCTTCATGGCCTCGGCCAGCCGCACGCTCATCTCGAAGTACTCGGCGTAGGT |
| FL-OR4 | GGGCATGAAGAACTGCAGGCTGTTCTCGCTGCACACCACGATCCGGTGGTTGGTGTTCAG |
| FL-OR5 | CTCGTTGTAGATGTCGTTGGCGGGGGCCACGGCCACGCCGATGAACAGGGCGCCCAGCAC |
| FL-OR6 | CTTCTTGCTCACGAACACCACGGTGGGCTGGCTGATGCCCATGCTGTTCAGCAGCTCCCG |
| FL-OR7 | GATGATGATCTTCTGGATGATGGGCAGCTTCTTCTGCACGTTCAGGATCTTCTGCAGGCC |
| FL-OR8 | GGGCAGGTGGCTGGTCACGAAGGTGTACATGCTCTGGAAGCCCTGGTAGTCGGTCTTGCT GTCCAT |
| FL-OR9 | GGTCTTGTCCCGGTCGAAGCTCTCGGGCACGAAGTCGTACTCGTTGAAGCCGGG |
| FL-OR10 | GGGCAGGGCCACGCCCTTGGGCAGGCCGGTGCTGCCGCTGCTGTTCATGATCAGGGCGAT |
| FL-OR11 | GATCTGGTTGCCGAAGATGGGGTCCCGGGCGTGGCTGAACCGCACGCAGGCGGTCCGGTG |
| FL-OR12 | GGTGAACATGCCGAAGCCGTGGTGGAAGGGCACCACGCTCAGGATGGCGGTGTCGGGGAT |
| FL-OR13 | CTCCTCCTCGAACCGGTACATCAGCACCACCCGGAAGCCGCAGATCAGGTAGCCCAGGGT |
| FL-OR14 | CAGGGTGGGCACCAGCAGGGCGCTCTGGATCTTGTAGTCCTGCAGGCTCCGCAGGAACAG |
| FL-OR15 | GGGCGCCGCCGCTGGCGATCTCGTGCAGGTTGCTCAGGTCGTACTTGTCGATCAGGGTGCTCTTGGCGAAG AAGCTGAA |

| | |
|---|---|
| FL-OR16 | GAACCGCTTGGCCACGGCCTCGCCCACCTCCTTGCTCAGGG |
| FL-OR17 | GATCAGGATGGCGCTGGTGGTCTCGGTCAGGCCGTAGCCCTGCCGGATGCCGGGCAGGTG |
| FL-OR18 | GGCCTCGAAGAAGGGCACCACCTTGCCCACGGCGCCGGGCTTGTCGTCGCCCTCGGGGGT |
| FL-OR19 | GCACAGCTCGCCCCGCTGGTTCACGCCCAGGGTCTTGCCGGTGTCCAGGTCCACCACCTT |
| FL-OR20 | CAGGGCGTTGGTGGCCTCGGGGTTGTTCACGTAGCCGCTCATGATCATGGGGCCCCGCAC |
| FL-OR21 | GTGCTCGTCCTCGTCCCAGTAGGCGATGTCGCCGCTGTGCAGCCAGCCGTCCTTGTCGAT |
| FL-OR22 | GGGGGCCACCTGGTAGCCCTTGTACTTGATCAGGCTCTTCAGCCGGTCCACGATGAAGAA |
| FL-OR23 | GCCGGCCACGCCGGCGTCGAAGATGTTGGGGTGCTGCAGCAGGATGCTCTCCAGCTCGGC |
| FL-OR24 | CTTGCCGTGCTCCAGCACCACCACGGCGGCGGGCAGCTCGCCGGCGTCGTCGTCGGGCAG |
| FL-OR25 | CTTCTTGGCGGTGGTCACCTGGCTGGCCACGTAGTCCACGATCTCCTTCTCGGTCATGGT |
| FL-OR26 | GTCCAGCTTGCCGGTCAGGCCCTTGGGCACCTCGTCCACGAACACCACGCCGCCCCGCAG |
| FL-OR27 | TCACACGGCGATCTTGCCGCCCTTCTTGGCCTTGATCAGGATCTCCCGGATCTTCCGGGC |
| Inducer DNAs (ORF region of GFP mRNA) | |
| OR1-L7nt | GTCCAGCTCGACCAGGACACCCCGGTGAACAGC |
| OR3-L7nt | GGTGGGCCAGGGCACGGGCAGCTTGCCGGGATGAACTTCAGGGTCAGCTTGCC |
| OR5-L7nt | GAAGATGGTGCGCTCCTGGACGCGGGCATGGCGGACTTGAAGAAGTCGTGCTG |
| OR7-L7nt | GTGCCCCAGGATGTTGCCGTCCTCCTTGTGCCCTTCAGCTCGATGCGGTTCAC |
| OR9-L7nt | GGCGAGCTGCACGCTGCCGTCCTCTGGCGGATCTTGAAGTTCACCTTGATGCC |
| OR11-L7nt | GACCATGTGATCGCGCTTCTCGTTGGGCTCAGGGCGGACTGGGTGCTCAGGTA |
| Inducer DNAs (ORF region of GFP mRNA) | |
| FL-OR8-L7nt | GGGCAGGTGGCTGGTCACGAAGGTGTACATGCTCCCTGGTAGTCGGTCTTGCT GTCCAT |
| FL-OR17-L7nt | GATCAGGATGGCGCTGGTGGTCTCGGTCAGGCCTGCCGGATGCCGGGCAGGTG |
| Primer pairs for RT-qPCR (ORF region of GFP mRNA) | |
| OR1-PP | fp: ACCATGGTGAGCAAGGG<br>rp: CTGAACTTGTGGCCGTTTAC |
| OR3-PP | fp: ATGCCACCTACGGCAAG<br>rp: TAGCGGCTGAAGCACTG |
| OR7-PP | fp: AAGTTCGAGGGCGACAC<br>rp: ATAGACGTTGTGGCTGTTGT |
| OR11-PP | fp: CCCGACAACCACTACCTGAG<br>rp: TCCATGCCGAGAGTGATCC |
| Primers for RT-stop analysis (ORF region) | |
| FAM-RT-OR7 | /FAM/AGCTTGTGCCCCAGGATG |
| Oligos of short RNA model test | |
| Short RNA | AUCCUGCCGACUACGCCA |
| DNA-L3nt | AAATGGCGTAGGCAGGATAAA |
| DNA-L5nt | AAATGGCGTACAGGATAAA |
| DNA-L7nt | AAATGGCGTAGGATAAA |

## Additional Figures

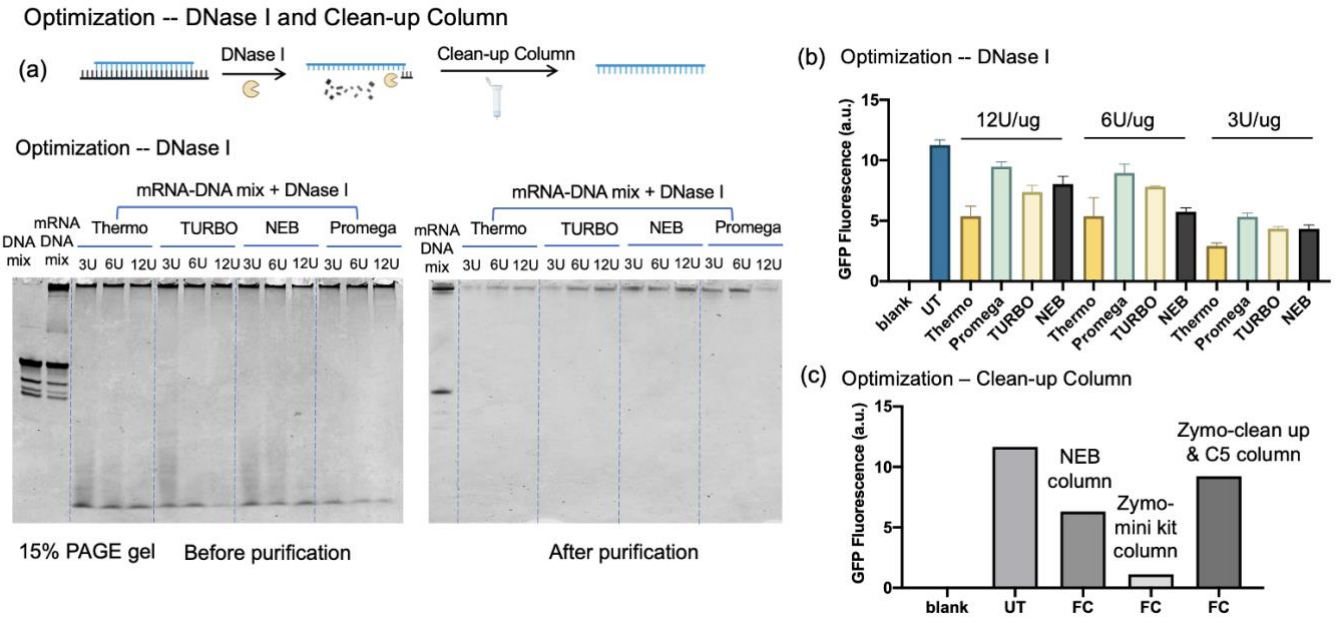



**Figure S1.** Optimization of tiled DNA removal for mRNA purification after TRAIL reaction. (a) Optimization of DNase I conditions by PAGE gel analysis of mRNA fully tiled with complementary DNAs and treated with DNase I for 1 h at 37 ℃. DNase I from different sources (Thermo, TUBO, NEB and Promega) were tested with different amounts of enzyme (3U, 6U, 12U). The DNase I from Promega showed the best catalytic activity in these experiments and the 6U amount was optimal for DNA digestion. After column purification, no DNA fragments were observed in the gel indicating that digested DNA fragments could be removed from mRNA with this purification method. (b) Optimization of DNase I to evaluate the effects on mRNA function, measured by in vitro translation of purified mRNA. mRNA treated with 6U or 12U Promega DNase I worked best, displaying similar expression levels as untreated mRNA, while other conditions showed reduced expression, which may be due to the presence of undetected small DNA fragments. (c) Optimization of clean-up column purification. After 6U Promega DNase I treatment, mRNA-DNA fragment mixtures were purified with different clean-up columns; the clean-up column and concentrator-5 from Zymo provided the best recovery of mRNA under these conditions. Thermo: Thermo Scientific™ DNase I, RNase-free (EN0525); TURBO: TURBO™ DNase, Invitrogen (AM2238); NEB: DNase I (RNase-free), New England BioLabs (M0303S); Promega: RQ1 RNase-Free DNase, Promega (M6101).

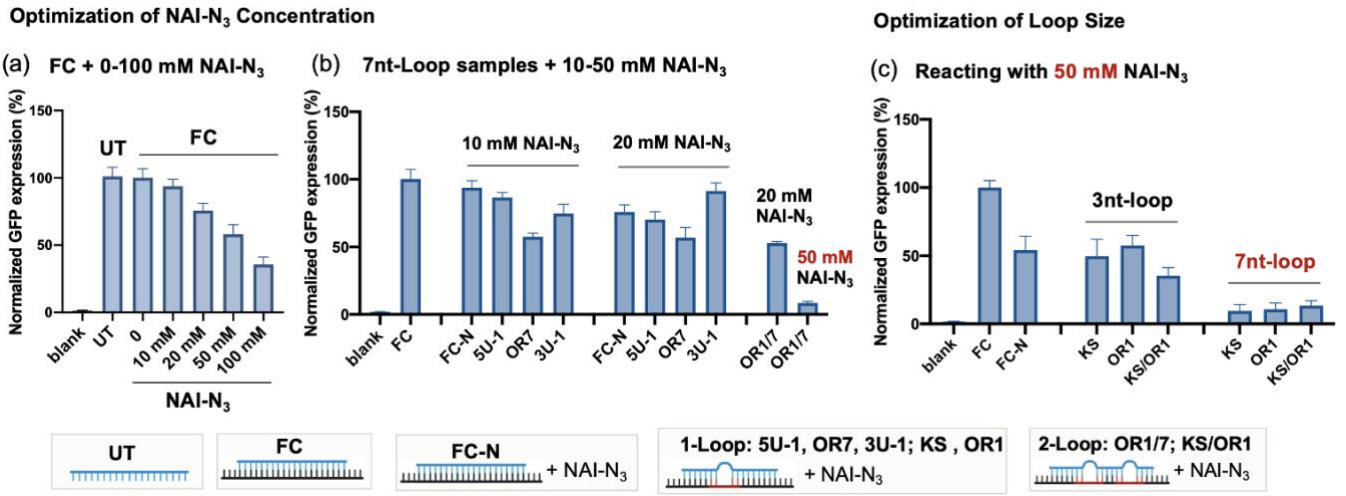



**Figure S2.** Optimization of NAI-$N_3$ concentration and induced loop size for high-yield acylation. (a) DNA tiling effects on protecting mRNA from acylation in different NAI-$N_3$ concentrations. mRNAs fully tiled with complementary DNAs (FC) were reacted with 0-100 mM NAI-$N_3$ and purified for in vitro translation. A small to moderate degree of translation inhibition was observed with increasing NAI-$N_3$ concentration, which might be due to the off-target acylation caused by DNA inducing alternative secondary structures in fully tiled mRNA-DNA duplex. (b) Site-selective acylation of mRNA with 10-50 mM NAI-$N_3$. mRNAs induced with one 7nt-loop (5'UTR: 5U-1, ORF: OR7, 3'UTR: 3U-1) and two 7nt-loops (ORF: OR1/7) were tested. 50 mM NAI-$N_3$ could not only efficiently suppress the ORF-acylated mRNA but also retain most of the translation ability of UTR-acylated mRNA and FC-RNA reacted with NAI-$N_3$ (FC-N), which was optimal for further experiments. (c) Loop size effect on site-selective acylation of mRNA. ORF-acylated mRNAs with one loop site (KS, OR1) and two loop sites (KS/OR1) were utilized for testing. 3nt-loop size and 7nt-loop size were constructed at each same site of mRNA, respectively. The results showed that ORF-acylated mRNA with 7nt-loop size could repress translation effectively while 3nt-loop size did not, indicating a higher-yield acylation at the larger loop size.

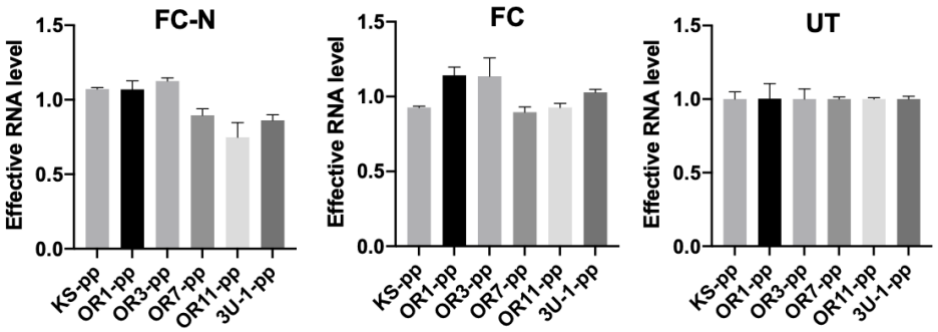



**Figure S3.** Evaluating primer pairs for probing prospective acylation sites. RT-qPCR analysis of untreated mRNA (UT), mRNA purified from fully complementary DNAs via tiling (FC) and FC treated with NAI-$N_3$ (FC-N). The effective RNA level of FC and FC-N were mostly similar with UT (which was normalized to "1" for each primer pair). FC-N with OR11-PP and 3U-1-PP showed a small decrease in amplifiable quantity, possibly from a low level of off-target acylation.

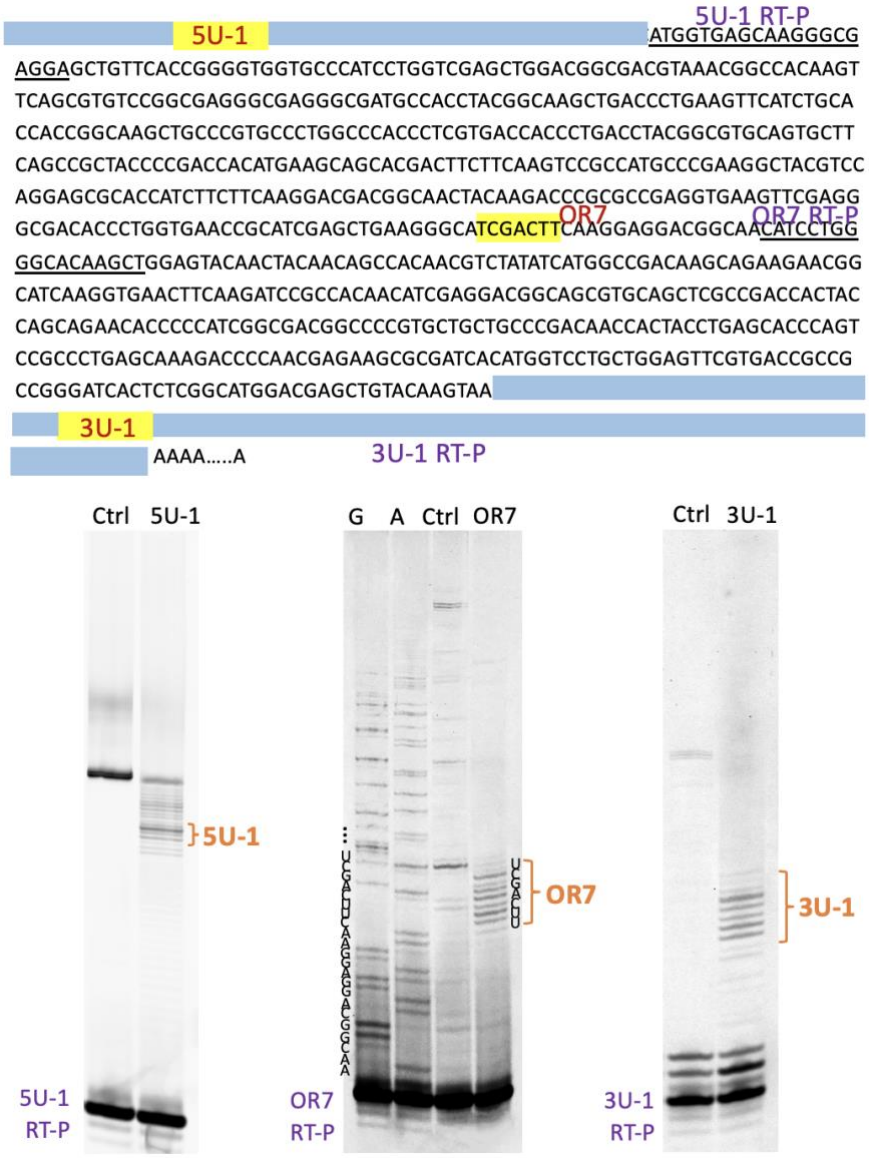



**Figure S4.** PAGE analysis of RT stops for site-selectively acylated mRNA at different loop-induced sites (5U-1, OR7, 3U-1), showing the degree of loop selectivity relative to protected RNA surrounding the site. The acylation sites and corresponding RT primers are shown above. The darker bands appear exactly at the sequence of the designed loop site for each acylated mRNA, indicating that acylation selectively occurs at the intended position on the mRNA. The middle gel (OR7) is the same one as shown in figure 2E.

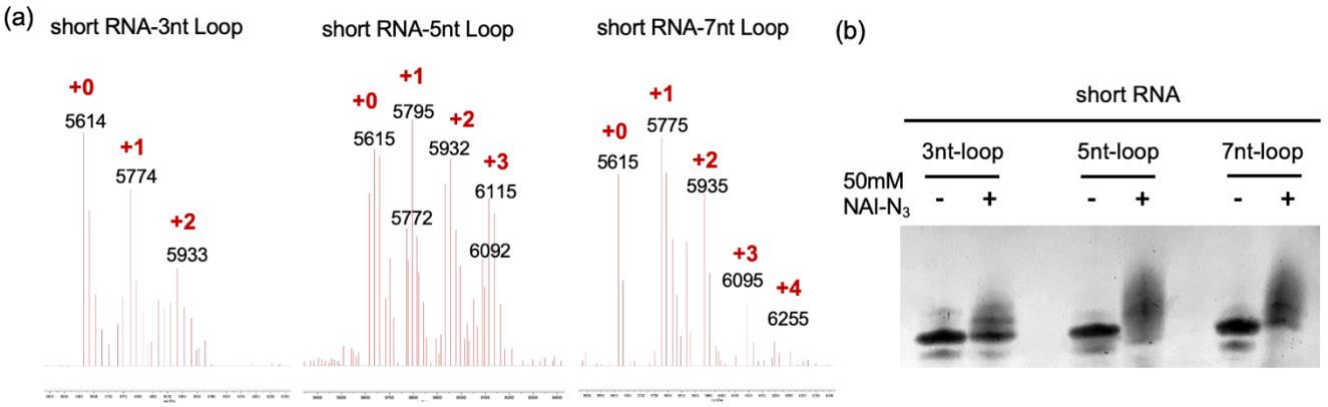



**Figure S5.** Evaluation of the number of acyl groups in induced loop sites by a short RNA model study. (a) MALDI-TOF mass spectra of DNA-induced 3nt-loop RNA, 5nt-loop RNA, 7nt-loop RNA reacting with 50mM NAI-$N_3$. Red numbers indicate the number of acyl groups per RNA, showing an average of 1 acyl group in 3nt-loop, 1-2 acyl groups in 5nt-loop and 2-3 acyl groups in 7nt-loop. (b) PAGE gel (15%) analysis of short RNAs acylated with different loop sizes (3nt, 5nt and 7nt)

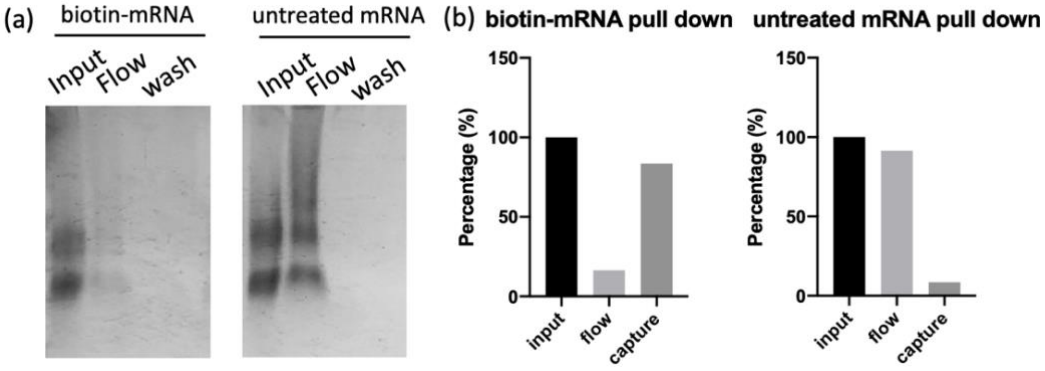



**Figure S6.** Evaluation of site-selective acylation efficiency via pull-down of acylated and further biotin labeled mRNA. (a) Agarose gel analysis of biotin-mRNA and untreated mRNA after pull-down by streptavidin beads. Input: original amount of mRNA for pull-down; Flow: remaining mRNA in the flow solution after pull-down; Wash: wash solution of the beads after pull-down. (b) Quantitative results of (a) by ImageJ software. The percentages of captured mRNAs were ~80% for biotinylated mRNA and ~10% for untreated mRNA due to non-specific binding, which indicates ~70% efficiency of acylation via TRAIL.

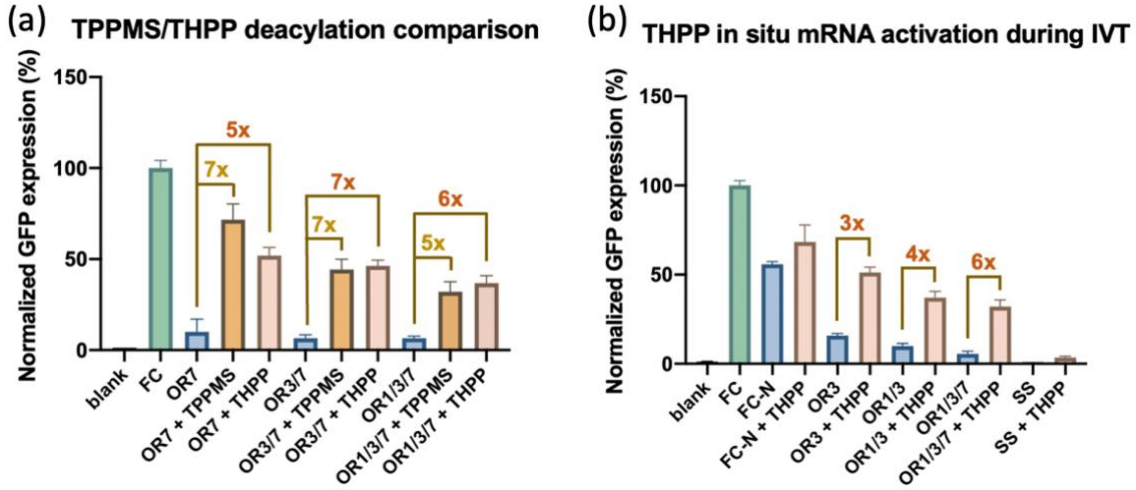



**Figure S7.** Reversing the acyl group substitution on mRNA via phosphines as evaluated by *in vitro* translation. (a) Comparison of phosphine deacylation ability with 3 mM TPPMS or THPP at 37 ℃ for 3 h. The results were measured by *in vitro* translation (IVT) of acylated and deacylated mRNAs separately. The data show that both phosphines exhibit similar activity for adduct removal, restoring translation ability of the mRNA effectively. (b) In situ activation of ORF-acylated mRNA with THPP during *in vitro* translation, de-acylating RNA directly in the translation mixture. 3 mM THPP was added to the wheat-germ translation system containing acylated mRNA, and GFP fluorescence was measured directly. The results revealed a ~3-5-fold activation of ORF-acylated mRNA and ~30%-50% expression relative to FC after *in situ* THPP treatment, which indicates compatibility of THPP in the WGE translation system.

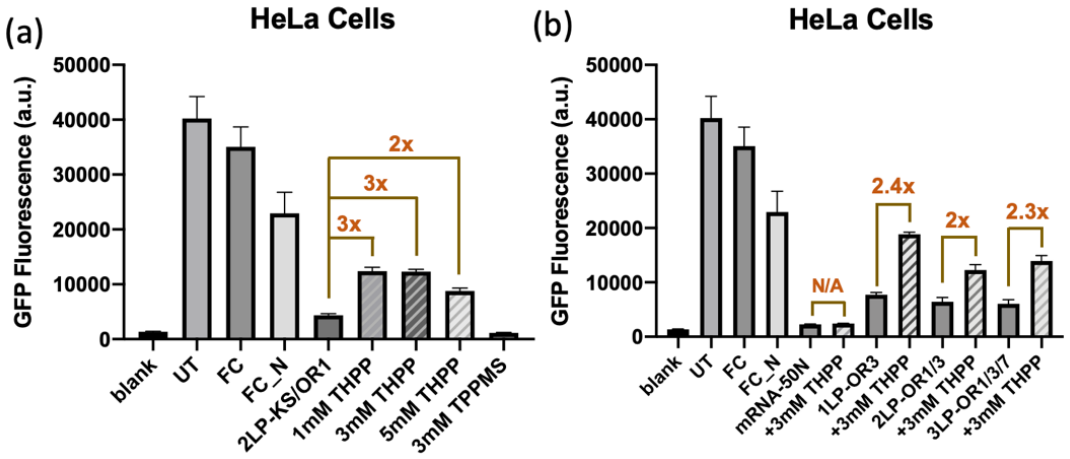
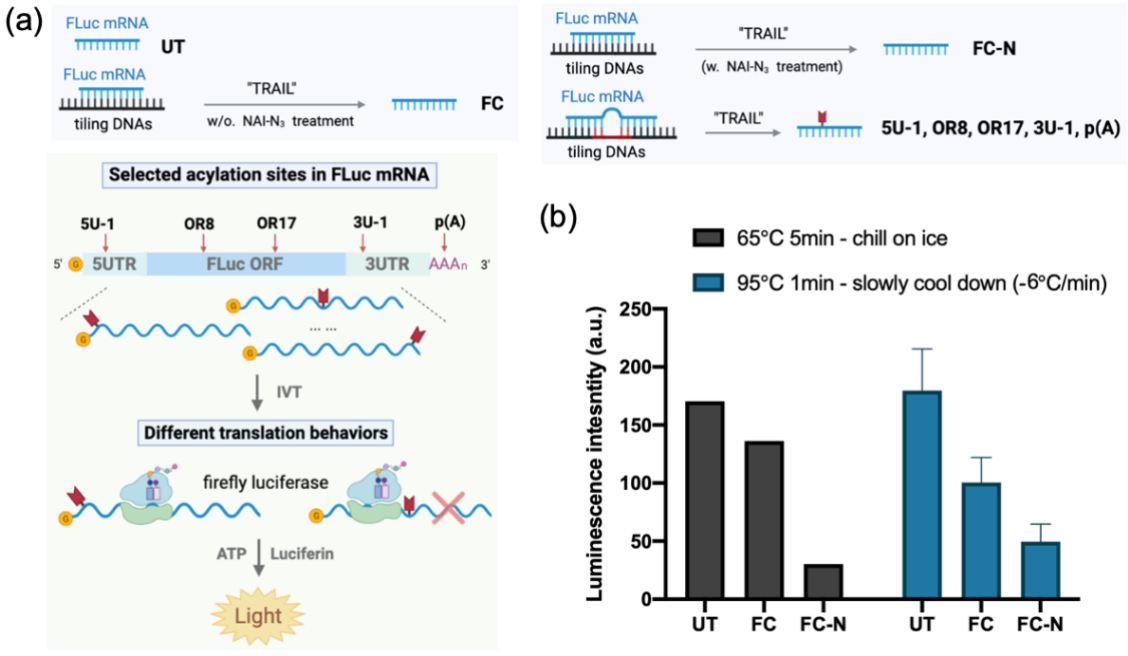
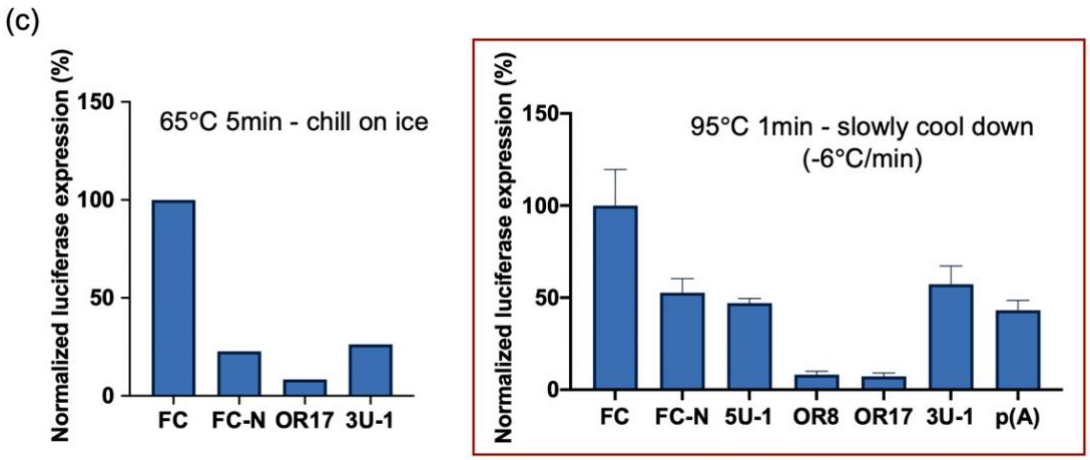



**Figure S8.** Phosphine-mediated activation of mRNA translation in HeLa cells. Cells were transfected with ORF-acylated GFP mRNA for 4 h, then treated with 1-5 mM phosphine for 1 h and further incubated for 3 h before fluorescence detection by a plate reader. (a) Testing varied concentrations of phosphines added to the cells for activation of mRNA acylated at 2 loop sites (2LP-KS/OR1). (b) Activation of different loop sites acylated mRNA (1LP-OR3, 2LP-OR1/3, 3LP-OR1/3/7) with 3 mM THPP in HeLa cells.



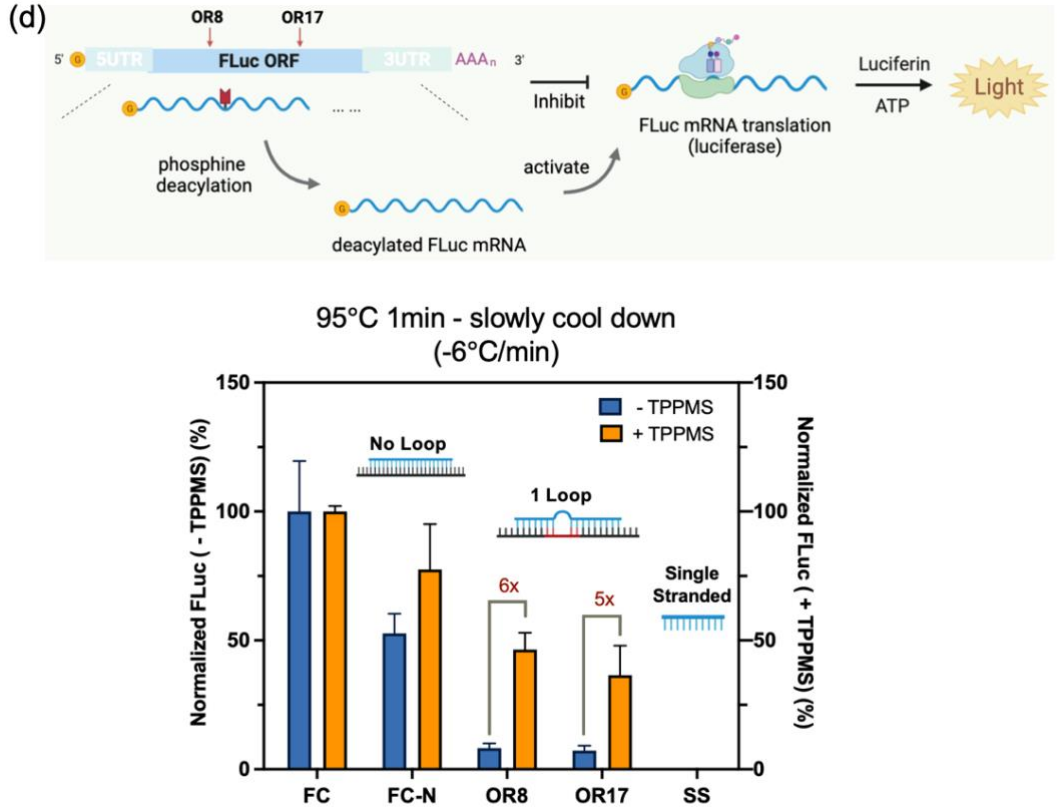



**Figure S9.** Application of TRAIL-mediated acylation to 1929nt firefly luciferase (FLuc) mRNA. (a) Schematics of the application of TRAIL method to FLuc mRNA. (b) In vitro translation of FLuc mRNA after TRAIL with different hybridization conditions. UT: untreated FLuc mRNA, FC: FLuc mRNA purified from fully complementary DNAs via tiling (FC), FC-N: FC treated with NAI-$N_3$. Although the high temperature annealing condition moderately impaired mRNA activity, the result showed improved protection of the long mRNA, apparently due to more efficient hybridization of the protector DNAs. (c) In vitro translation of selectively acylated FLuc mRNA (5U-1, KS, OR1, OR3, OR7, OR11, 3U-1) at different sites from 5'UTR to 3'UTR, showing strong suppression of translation at ORF sites similar as GFP mRNA. FC was employed as a positive control and its FLuc expression level was normalized to 100%. (d) In vitro translation of ORF-acylated FLuc mRNAs and deacylated mRNA restored by treating with 3mM TPPMS.

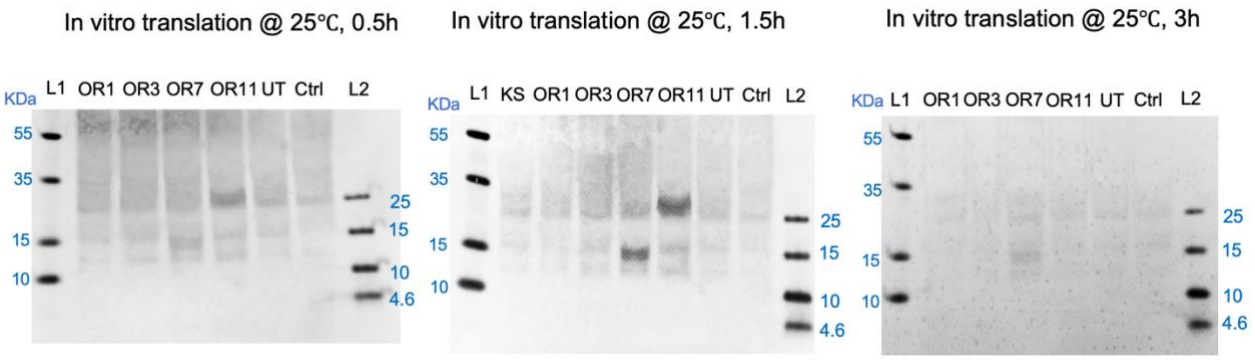



**Figure S10.** Optimization of translation incubation time for analysis of stalled peptides detected by Western blot. 1.5 h incubation of *in vitro* translation with ORF-acylated mRNA showed the clearest peptide bands in the membrane. We hypothesize that lower intensity of bands at longer times may be due to rescue factors in the extract that dissociate ribosomes from the RNA[3]. The middle image is the duplicate in Figure 4B.

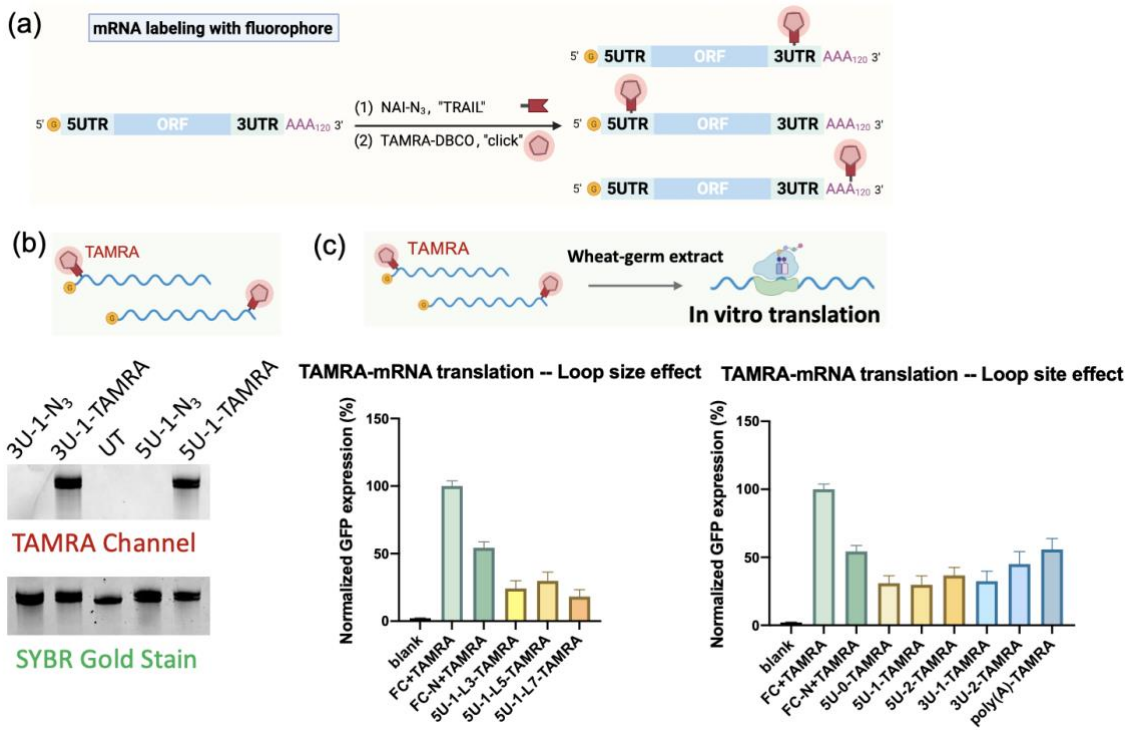



**Figure S11.** mRNA labeling with fluorophore TAMRA via TRAIL at UTR sites of GFP mRNA. (a) Schematic of fluorophore labeling via TRAIL and further click conjugation chemistry. (b) Agarose gel analysis showing efficient TAMRA labeling of mRNA. (c) Loop size and labeling sites effect of TAMRA labeled mRNA evaluated by in vitro translation. A 5nt-loop size was chosen for mRNA fluorophore labeling. The translation ability of TAMRA labeled mRNA at different UTR sites and poly(A) tails was retained, and the expression level was ~30-55% of non-acylated mRNA.

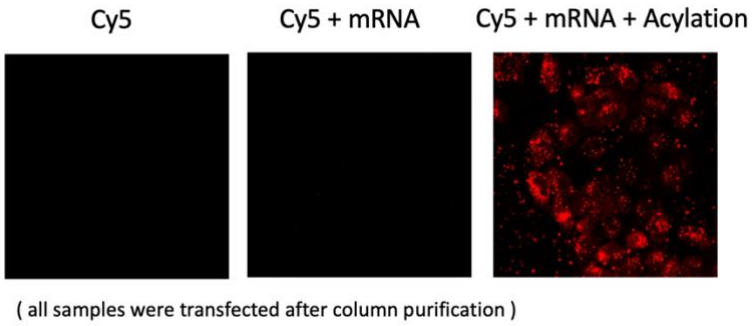



**Figure S12.** Imaging fluorophore-labeled mRNA in cells using TRAIL labeling. Confocal images of HeLa cells are shown; cells were transfected with column-purified Cy5 dye alone (Cy5), Cy5 dye incubated with untreated mRNA (Cy5 + mRNA) and Cy5 dye incubated with acylated mRNA (Cy5 + mRNA + Acylation). The results confirm covalent labeling of the RNA. RNA was labeled at the 5U-1 site as described in Figure 6.

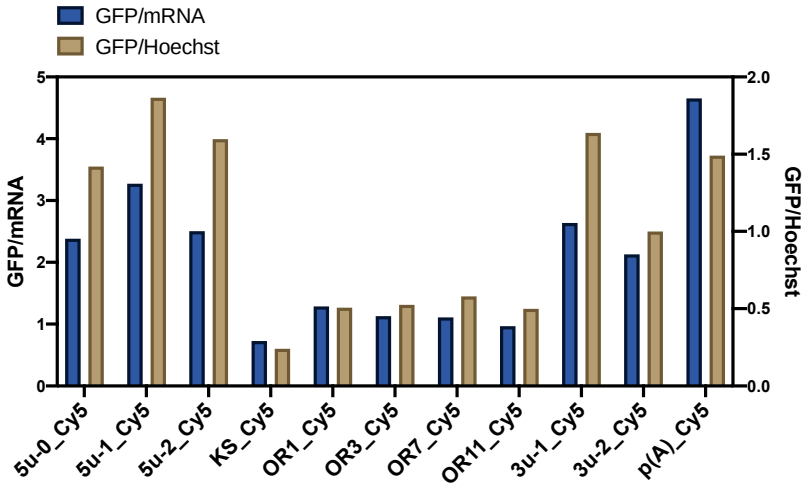



**Figure S13.** Statistics of the fluorescence intensity ratios of GFP/mRNA and GFP/ Hoechst in Figure 6c. The fluorescence intensity was quantified by Image-J.

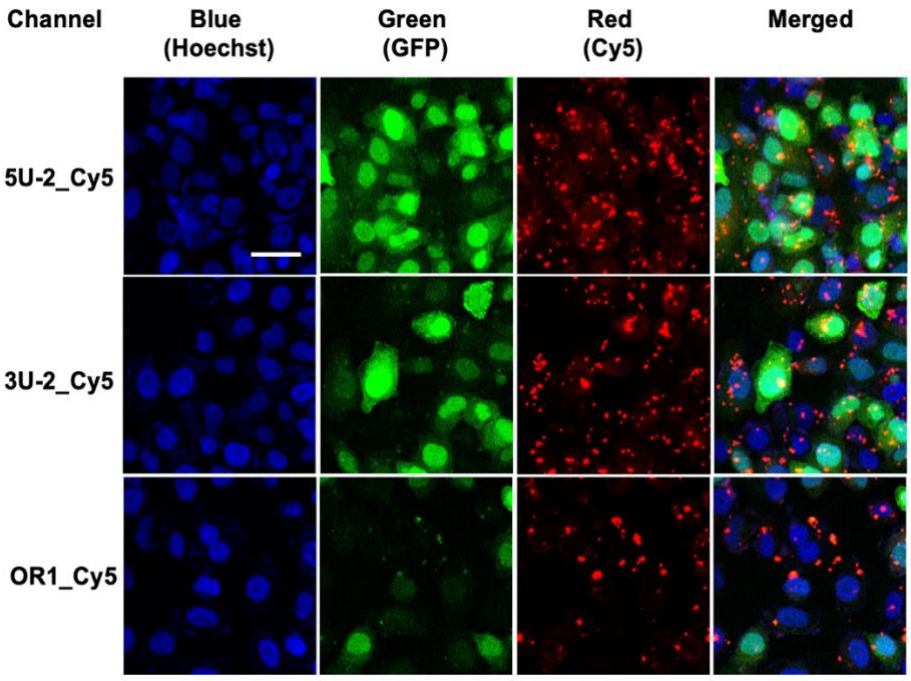



**Figure S14.** Enlarged three-channel images of Cy5-labeled mRNA (labeled via TRAIL; 5U-2_Cy5, 3U-2_Cy5, OR1_Cy5) transfected into HeLa cells. Blue: nuclei stained by Hoechst 33342; Green: translated GFP protein; Red: Cy5-labeled mRNA. Scale bar: 50 μm.

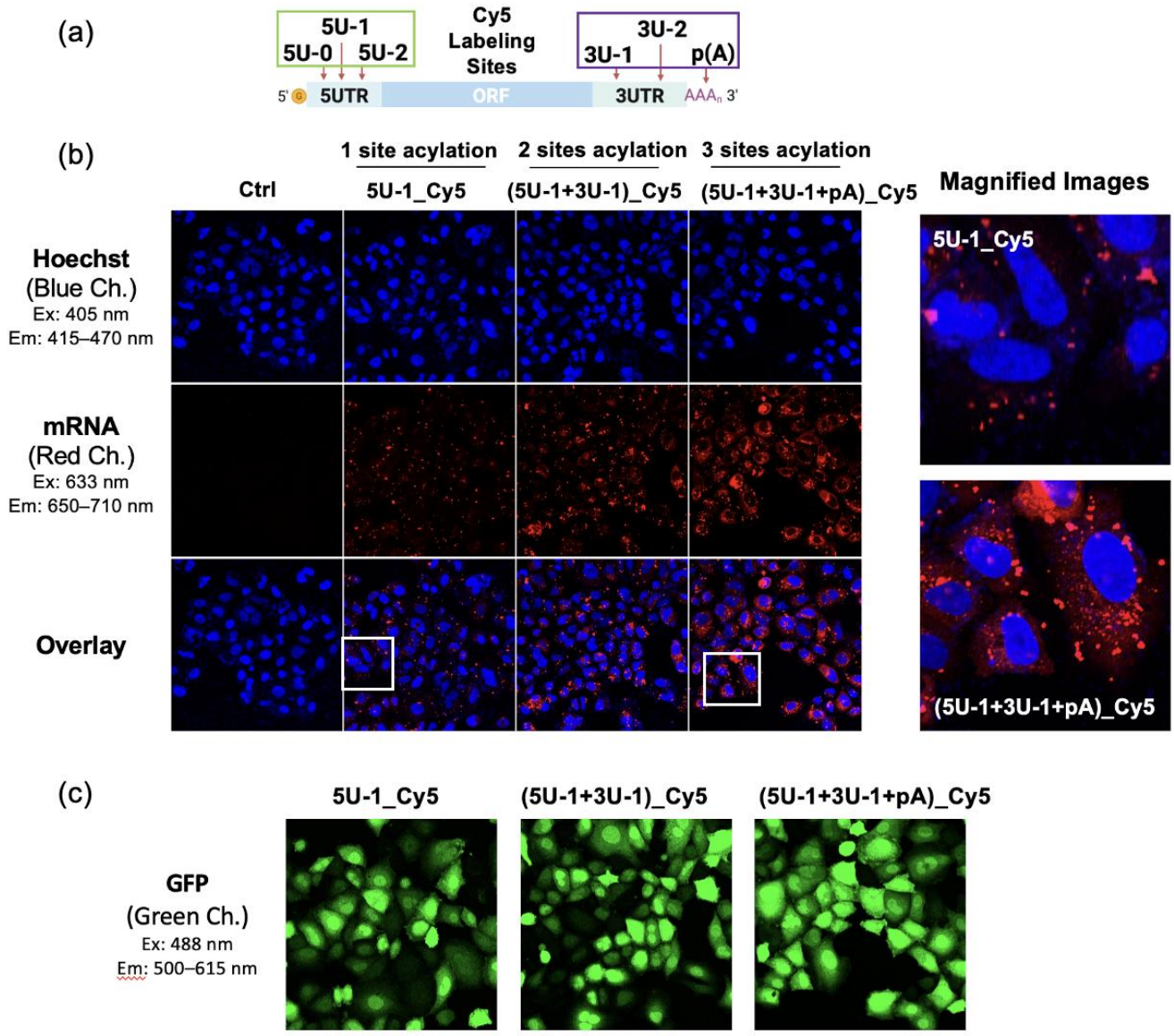



**Figure S15.** Imaging and translation of Cy5-labeled mRNA in several UTR sites (labeled via TRAIL) transfected into HeLa cells. The translation was measured by the fluorescence intensity of GFP protein, and mRNA was visualized by labeled Cy5 dye (red) using confocal microscopy after 4 h incubation. 5U-1_Cy5: 1 site acylation, (5U-1+3U-1)_Cy5: 2 sites acylation, (5U-1+3U-1+pA)_Cy5: 3 sites acylation. (a) Schematics of the Cy5 labeling sites in the mRNA. (b) and (c) Images of cells transfected with Cy5-labeled mRNAs at several sites in 5UTR or/and 3UTR.

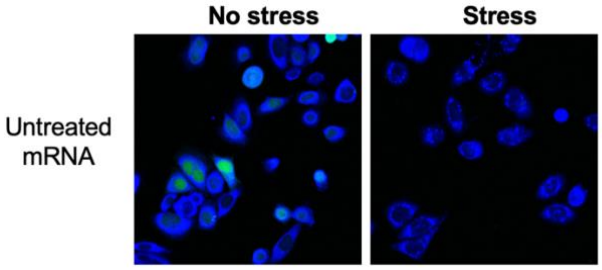



**Figure S16.** Translation of untreated mRNA under oxidative stress conditions as a control. Immunofluorescence images of untreated mRNA in the presence/absence of stress. 200 μM sodium arsenite was first added to the cells as an oxidative stress to induce SGs, and mRNA was transfected 30 min later without removal of arsenite from the medium. No GFP expression observed under the stress conditions. Blue: G3BP1-labeled SGs via immunostaining; Green: GFP protein; Red: Cy5-labeled mRNA.

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[2] A. C. Panda, J. L. Martindale, M. Gorospe, *Bio-protocol* **2016**, *6*, e2062.

[3] A. R. Buskirk, R. Green, *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **2017**, *372*, 20160183.

## Author Contributions

L. Xiao developed the TRAIL method and collected data, and wrote the manuscript. Y. W. Jun carried out the cell imaging tests and contributed to the writing. Prof. Dr. E. T. Kool led the project as PI and contributed to writing of the manuscript.