Chapter 9

Measuring Proximity-Mediated Function of mRNA Regulatory Proteins by Engineered Tethering

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Abstract

A powerful approach for studying the functional consequences of site-specific RNA–protein interactions is to artificially tether a protein to a messenger (or noncoding) RNA through a selective, high-affinity interaction. We share a strategy for evaluating the contribution of protein positioning within an mRNA on gene expression. We introduced an RNA hairpin recognition site for the MS2 coat protein into the untranslated regions or coding sequence of mRNAs expressing a luminescent reporter protein, NanoLuc. Effector proteins fused to the MS2 coat protein could thus be targeted to distinct regions across the mRNA. We illustrate this approach using ZFP36L2, which recruits the CCR4-NOT complex for poly(A) tail deadenylation. Tethering ZFP36L2 to the 3'-UTR decreased NanoLuc expression, as expected, given the known interaction of this adapter protein with adenine uridine-rich elements (AREs). Intriguingly, ZFP36L2 also decreased NanoLuc expression when bound within the coding sequence, revealing that ZFP36L2—and potentially many other mRNA regulatory proteins—can function when targeted to diverse locations within an mRNA. This multi-target tethering strategy enables exploration of the interplay between mRNA–protein proximity and gene expression.

Key words RNA-protein interactions, RNA tethering, mRNA proximity, RNA-binding proteins, Dual-luciferase assay, MS2 hairpin, MS2 coat protein

1 Introduction

Regulation of messenger RNA (mRNA) function, from transcription to degradation, involves hundreds of regulatory RNA-binding proteins [1, 2]. Many mRNA regulatory proteins are recruited to a specific location within an mRNA. For example, zinc finger proteins are recruited to 3′ untranslated regions (3′-UTRs) through binding to AU-rich elements (AREs) [3]. Cap-binding proteins bind the mRNA 5′-cap to promote translation and prevent decapping and thus 5′-to-3′ degradation [4]. However, the role that target site location plays in the function of proteins that regulate mRNA biology remains poorly understood. In this chapter, we describe a strategy for site-specific tethering of an mRNA

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regulatory protein to a particular region within an mRNA to investigate how binding at a specific site on the mRNA influences the ability of a protein to effect gene expression.

To site specifically tether a protein to an RNA, we took advantage of the highly specific and high-affinity interaction between the bacteriophage MS2 coat protein and an RNA hairpin binding site [5]. An mRNA-regulating "Effector" protein is fused to the MS2 coat protein (the tethering protein, TP) and targeted to a specific site in the RNA of interest using the RNA target hairpin (TH) at desired sites. In the assay we describe here, the target mRNA expresses NanoLuc, a bioluminescent protein [6]. The NanoLuc signal is correlated with the expression of the target mRNA. It is normalized to the signal from firefly luciferase [7], a second bioluminescent protein expressed in the same cell. If binding by the Effector-TP fusion changes target mRNA expression levels, light output is altered relative to a control expressing the TP alone or a TP fusion with a non-functional control protein (we use superfolder green fluorescent protein, sfGFP) (Fig. 1).

We created a set of plasmids encoding target mRNAs containing a single target hairpin (TH) at one of four sites: the 5′-UTR, the coding region (CDS, two sites), or the 3′-UTR. An mRNA omitting the TH serves as a control (Fig. 2a). In the coding region, the TH sequence encodes amino acids with a neutral hydropathy index (Fig. 2b) and is positioned within beta-hairpin turns that face away from the NanoLuc protein active site (Fig. 2c) to support correct protein folding and retain NanoLuc enzyme activity.

Dual-luciferase assays require optimization and careful execution. First, all plasmids must be carefully validated for integrity and have a supercoiled topology (Fig. 3). Second, plasmids encoding the target mRNA (NanoLuc) and firefly luciferase control must be transfected at levels yielding a linear relationship between plasmid amount and measured luminescent signals (Fig. 4a). In particular, expression at high levels can lead to protein precipitation or loss of activity. The plasmids developed here yield broad linear expression ranges in HEK293 cells (Fig. 4b). Third, luminescence must be measured when the amount of expressed luciferase protein is proportional to light production. Waiting too long to measure luminescence after plasmid transfection can allow NanoLuc to accumulate to cytotoxic levels (Fig. 4a). Transfection conditions for the firefly luciferase control plasmid and for the target mRNA plasmid should be optimized before the introduction of the plasmid expressing the Effector-TP fusion protein.

To evaluate the effect of site-specific binding of an Effector-TP fusion on target mRNA expression, the three plasmids are co-transfected into cells and allowed to express their respective proteins (Fig. 1). Firefly control and target mRNA expression levels are subsequently quantified using sequential luminescence measurements for firefly luciferase and NanoLuc, respectively. Three

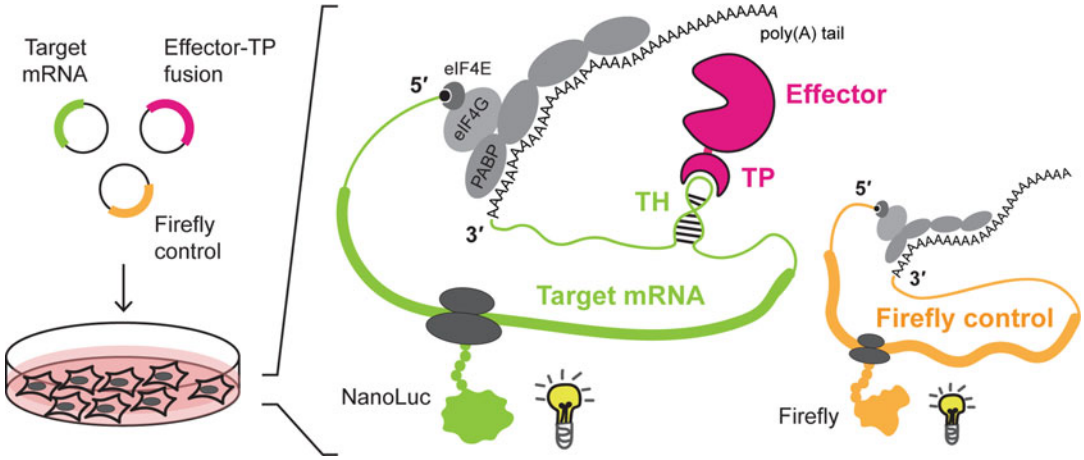

Fig. 1 Monitoring effects of site-specific binding of a protein to an mRNA via a luminescent reporter assay. Plasmids (3 total) expressing target mRNA with target hairpin (TH), Effector-tethering protein (TP) fusion, and firefly control are transfected into cells. The Effector-TP fusion protein binds the TH in the target mRNA. The signal due to NanoLuc, expressed from the target mRNA, is normalized to the signal due to firefly luciferase, expressed from the control plasmid. mRNAs are shown as lines; thicker regions indicate coding regions. Dark and light gray ovals indicate ribosomes and poly(A) binding proteins, respectively

calculations are then performed (Fig. 5): First, signals are corrected for background by subtracting the average signal from all wells containing cells but not transfected with plasmid. The NanoLuc signal, produced from the target mRNA, is divided by the firefly luciferase signal. This per-well normalization accounts for well-to-well differences in cell number, plasmid transfection efficiencies, and target mRNA expression. Second, data from triplicate wells are averaged. Third, the average normalized NanoLuc signal from Effector-TP fusion-expressing cells is divided by the signal from TP (only)-expressing cells to calculate a "fold change" value. This experiment ultimately reveals site-specific regulatory effects of the Effector protein, which may involve either a decrease or increase in protein production from the target mRNA.

2 Materials

2.1 Plasmids

1. Transfection carrier DNA (Promega).
2. pGL4.50 vector (Promega) encodes firefly control.
3. pNL3.2.CMV.NLuc-PEST.Hygro vector encodes target mRNA with no TH (no-TH control and parent vector for TH-containing mRNAs).
4. pNL3.2.CMV.5UTR-TH.NLuc-PEST.Hygro vector encodes target mRNA with TH in the 5'-UTR.

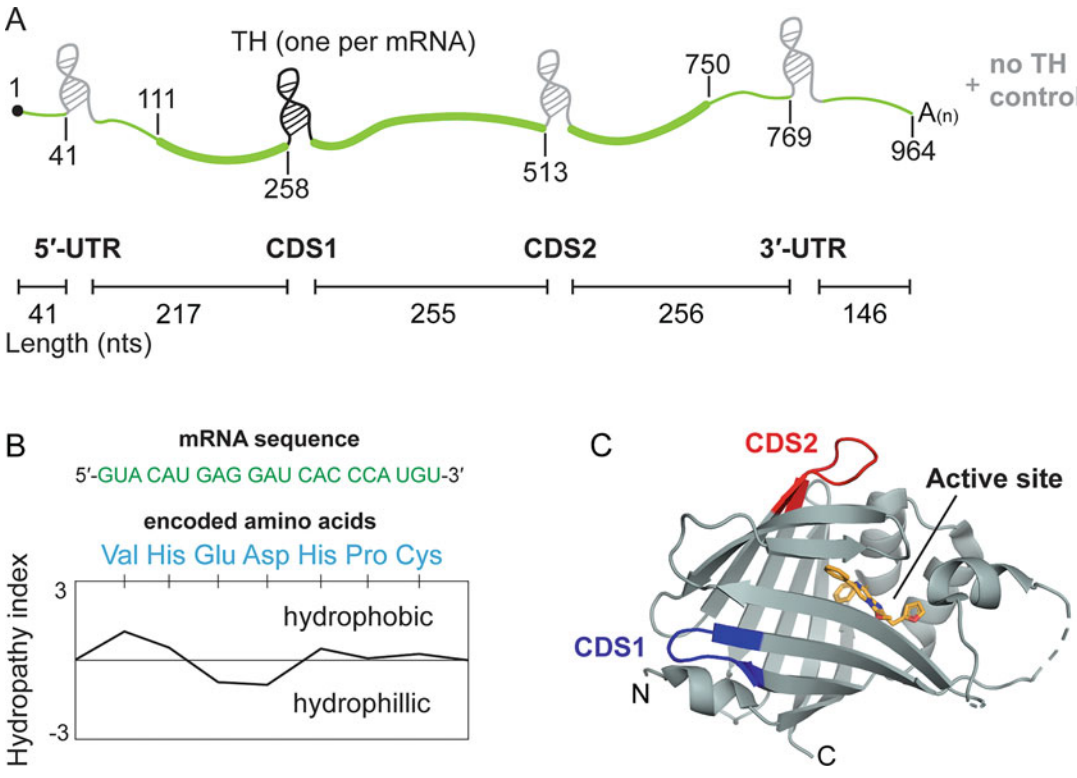

Fig. 2 Target mRNA design. (**a**) Four target mRNAs contain single target hairpins (TH) inserted at evenly spaced sites across the mRNA. An mRNA with no TH is used as a control. (**b**) Targeting hairpin sequence and hydropathy index for the encoded peptide. (**c**) Structure of NanoLuc with substrate analog in the active site (PDB 7SNT). Insertion sites at beta-hairpin turns, encoded by the CDS1 and CDS2 mRNAs, are indicated in blue and red, respectively. A single insertion occurs per mRNA

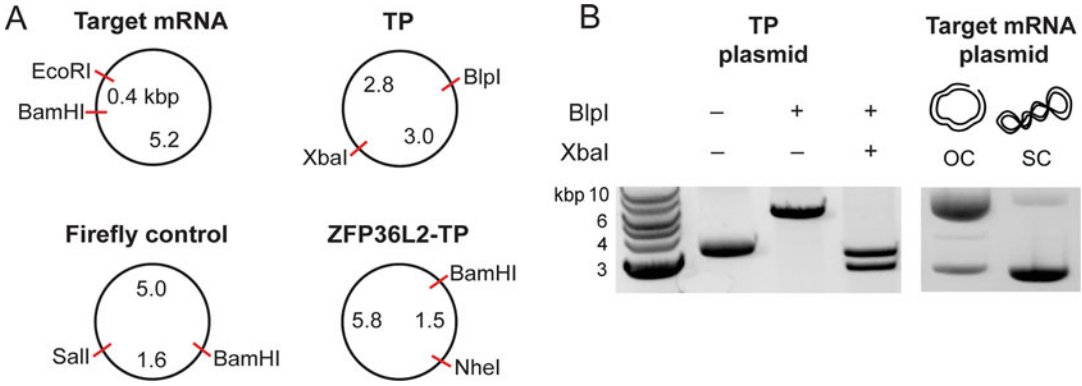

Fig. 3 Validation of plasmid size and topology. (**a**) Diagrams of useful restriction enzyme sites in assay plasmids. Numbers give expected band sizes in kbp. For single plasmid digestions, select one of the two indicated enzymes. (**b**) Plasmid quality control, visualized by agarose gel electrophoresis. Gels showing (*left*) DNA ladder, undigested TP plasmid, single and double digestions of the TP plasmid; and (*right*) no-TH target mRNA plasmid in nicked and supercoiled forms

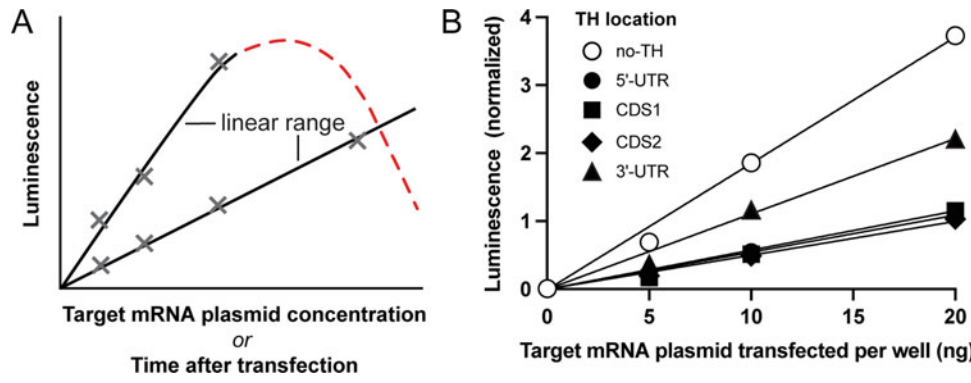

Fig. 4 Target mRNAs show broad linear expression ranges. (**a**) Schematic plot of luminescence as a function of target mRNA plasmid concentration (or time after transfection). Linear expression region shown in black; region of NanoLuc precipitation or cellular toxicity emphasized in red. (**b**) Representative experimental data showing linear expression for the five target mRNAs

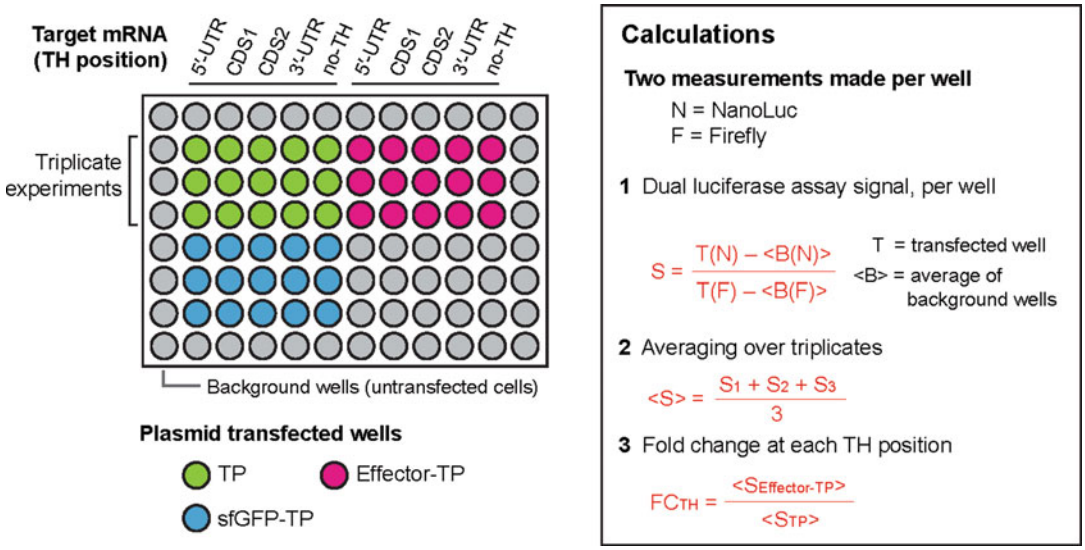

Fig. 5 Representative plate layout and calculations to determine effects of effector protein tethering. (*left*) Representative plate layout. Plate columns correspond to the target mRNA transfected. The well color indicates TP fusion protein transfected. All wells contain firefly plasmid. Samples are analyzed in triplicate. Wells containing untransfected cells are shown in gray. (*right*) Calculations to determine the effect of Effector-TP fusion protein on (NanoLuc expressing) target mRNAs

5. pNL3.2.CMV.CDS1-TH.NLuc-PEST.Hygro vector encodes target mRNA with TH in the CDS1 position.
6. pNL3.2.CMV.CDS2-TH.NLuc-PEST.Hygro vector encodes target mRNA with TH in the CDS2 position.
7. pNL3.2.CMV.3UTR-TH.NLuc-PEST.Hygro vector encodes target mRNA with TH in 3'-UTR.

8. pA.CBh.TP-V5H6.MCh.Puro encodes TP alone (negative control and parent vector for Effector-TP fusion proteins).
9. pA.CBh.TP-sfGFP-V5H6.MCh.Puro encodes sfGFP-TP fusion.
10. pA.CBh.TP-ZFP36L2-V5H6.MCh.Puro encodes ZFP36L2-TP fusion.

2.2 Plasmid Preparation

1. *E. coli* competent cells: any *E. coli* cloning strain with recA1 deficiency or mutation (*see* **Note 1**).
2. LB agar plates with 50 μg/ml ampicillin.
3. Sterilized (autoclaved) 500 ml cell culture flasks.
4. LB broth (Miller) with 50 μg/ml ampicillin.
5. Midiprep plasmid purification system that removes endotoxins.

2.3 Plasmid Quality Check

1. EcoRI-HF, SalI-HF, BamHI-HF, NheI-HF, XbaI, and BlpI enzymes.
2. Supplied restriction enzyme buffer(s).
3. Agarose.
4. Tris–borate–EDTA (TBE) buffer: weigh 10.8 g Tris base, 5.5 g boric acid, 4 ml of 500 mM EDTA (pH 8). Make up to 1 l with deionized water.
5. Ethidium bromide.
6. 1 kb DNA ladder.
7. DNA gel loading dye.
8. Nuclease-free water.

2.4 Mammalian Cell Preparation

1. HEK293 cells (ATCC CRL-1573).
2. Complete Dulbecco's modified Eagle's medium (DMEM), DMEM supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml).
3. 100 mm Tissue culture-treated dishes.
4. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4. Adjust pH to 7.4.
5. Trypsin with EDTA: 0.05% trypsin with 0.02% (0.53 mM) EDTA.
6. 10 ml Serological pipettes.

2.5 Plasmid Transfection

1. 0.2 ml 8-strip PCR tubes.
2. 1.6 ml Microcentrifuge tubes, autoclaved.
3. Fugene 6 transfection reagent (Promega).
4. DMEM.

5. Hemocytometer.
6. Microscope coverslips.
7. Trypan blue.
8. 25 ml Reagent reservoirs.
9. 96-Well, white, clear-bottom tissue culture-treated plate with lid.

2.6 Dual-Luciferase Assay

1. 25 ml Reagent reservoirs.
2. Nano-Glo Dual-Luciferase Reporter Assay System (Promega).

2.7 Instruments

1. Tabletop centrifuge.
2. Vacuum manifold.
3. Gel imager.
4. Agarose gel electrophoresis system.
5. Mammalian cell culture CO_2 incubator.
6. Mammalian cell culture biological safety cabinet.
7. Microscope.
8. NanoDrop UV-Vis spectrophotometer.
9. Qubit 2.0 fluorometer.
10. Luminescence microplate reader.

3 Methods

3.1 Plasmid Preparation

1. Transform plasmid DNA into *E. coli* competent cells, according to manufacturer technical protocol.
2. Spread transformed cells on individual LB agar plates containing 50 μg/ml ampicillin and incubate at 37 °C overnight until individual colonies grow.
3. Using a sterile pipette tip, inoculate 100 ml sterilized LB broth (Miller) and 50 μg/ml ampicillin in a sterilized 500 ml cell culture flask with a single bacterial colony.
4. Incubate flask in a shaking incubator at 37 °C and 400 rpm for 12 h.
5. Measure optical density (OD) at 600 nm every 2 h until OD remains constant and *E. coli* reach stationary phase of growth (*see* **Note 2**).
6. Harvest plasmids with Midiprep plasmid purification system according to manufacturer protocol.

3.2 Plasmid Quality Control

1. Quantify plasmid DNA using a spectrophotometer (*see* **Note 3**).
2. Confirm integrity and topology of plasmids by performing single and double restriction enzyme digestions on 1 μg of the plasmid. Useful enzymes for each plasmid are shown in Fig. 3a. Optimal digestion conditions can be obtained from the NEBcloner restriction digest website.
3. Prepare 0.8% (w/v) agarose gel with TBE as the running buffer [*see* ref. 8].
4. Add loading dye to the DNA ladder and all DNA samples.
5. Load the DNA ladder in the leftmost and rightmost lanes on the gel. Load 1 μg of undigested plasmid and plasmids digested with one or two enzymes in adjacent lanes on the gel.
6. Run the gel at 120 V until tracking dye is 75% of the way through the gel (about 1–1.5 h).
7. Image gel under 300 nm UV light (Fig. 3b, left).
8. Analyze undigested plasmid and plasmid digested with one or two enzymes and confirm that all plasmids are predominantly in supercoiled topology (*see* **Note 2**). Nicked and linearized plasmids migrate more slowly than (the required) supercoiled plasmid (Fig. 3b, right).

3.3 Preparation of Mammalian Cells

1. Perform all mammalian cell work in a mammalian cell culture biological safety cabinet and incubate mammalian cells in dedicated mammalian CO_2 incubator at 37 °C, supplemented with 5% CO_2.
2. Pre-warm complete DMEM to 37 °C.
3. Obtain HEK293 cells (stored at −80 °C in aliquots) and thaw in a sterile 37 °C water bath for 2 min (*see* **Note 4**).
4. Spin cells down at 250 × g for 5 min, remove supernatant, and resuspend the cell pellet in 1 ml of complete DMEM.
5. Place resuspended HEK293 cells in a 100 mm tissue culture dish containing 9 ml complete DMEM and gently swirl to mix.
6. Place the cell culture dish in a CO_2 incubator and grow HEK293 cells to 80% confluency.
7. Passage cells twice (passaging cells involves performing **steps 8–12**). *See* **Note 5**.
8. Aspirate medium from cell culture dish and wash cells twice in 1× PBS to remove cell debris.
9. Add 2 ml trypsin with EDTA to the cell culture dish and incubate for 5 min at 37 °C to detach cells.

10. Quench trypsin with EDTA with 8 ml complete DMEM and suspend remaining adherent cells into solution by washing cells off the surface with a serological pipette.
11. Add 2 ml of detached HEK293 cells to a clean cell culture dish and dilute with 8 ml complete DMEM to complete cell passage.
12. Place the cell culture dish in a CO_2 incubator and grow HEK293 cells to 80% confluency.

3.4 Plating Mammalian Cells for Dual-Luciferase Assay

1. Pre-warm complete DMEM to 37 °C.
2. Wash cells twice in 1× PBS, detach cells with 2 ml of trypsin with EDTA, and dilute in 8 ml of complete DMEM (*see* **Note 6**).
3. To quantify cell number, add 100 µl of detached HEK293 cells and 100 µl of trypan blue to a sterile 1.6 ml microcentrifuge tube and pipette to mix (*see* **Note 7**).
4. Add 20 µl of mix to a clean hemocytometer with a cover slip.
5. Count cells using a microscope with a 10× objective and dilute cells to a density of 100,000 cells per ml with complete DMEM.
6. Place diluted HEK293 cells (10 ml per 96-well plate) in a 25 ml reagent reservoir and add 100 µl of cells to each well in a 96-well plate using a P200 multi-channel pipette. Two 96-well plates are required for identifying target mRNA (NanoLuc) linear expression ranges.
7. Place 96-well plate(s) in a CO_2 incubator for 24 h.
8. Passage the remaining HEK293 cells and place them in a CO_2 incubator for future experiments. Cells can be passaged up to ten times before discarding.

3.5 Identifying Linear Expression Ranges for Firefly Control and Target mRNA

1. First, complete this section for the firefly luciferase control plasmid (**step 1**). Second, repeat this section for each of the five target mRNA plasmids, using the firefly luciferase plasmid concentration optimized in the first step (**step 2**).
2. Allow serum-free DMEM and Fugene 6 to equilibrate to room temperature.
3. Individually dilute firefly control plasmid (or target mRNA plasmids) to 70 ng/µl and carrier DNA to 200 ng/µl with nuclease-free water to total volumes of 400 µl.
4. Quantify plasmid concentrations using the Qubit instrument following the manufacturer protocol (*see* **Note 8**).
5. Aliquot 40 µl of the firefly control plasmid (or target mRNA plasmids) into a fresh 0.2 ml strip PCR tube.

6. Perform a serial dilution of the firefly control plasmid (or target mRNA plasmids) in twofold steps with nuclease-free water to create 1:2 and 1:4 dilutions from stock, respectively.
7. (In **step 1**) Use serial dilutions to add 0 ng, 100 ng (1:4 dilution), 200 ng (1:2 dilution), and 400 ng (stock) of firefly control plasmid to 0.2 ml strip PCR tubes as samples 1–4, respectively. Add 1000 ng, 900 ng, 800 ng, and 600 ng of carrier DNA to samples 1–4, respectively. Each sample will contain 1000 ng of total plasmid DNA. (In **step 2**) For each target mRNA plasmid, add 0 ng, 100 ng (1:4 dilution), 200 ng (1:2 dilution), and 400 ng (stock) to 0.2 ml strip PCR tubes as samples 1–4, respectively. Then, add optimized firefly control plasmid concentration from **step 1** to samples 1–4. Finally, add carrier DNA to samples 1–4 so each sample contains 1000 ng of plasmid DNA.
8. Bring all samples to 20 μl with nuclease-free water and mix by pipetting.
9. Add 4 μl of each sample to a clean 0.2 ml strip PCR tube, sterilize the outsides of tubes with ethanol, and bring it into the mammalian cell culture hood.
10. Prepare 6× Fugene 6:DNA (0.6 μL of Fugene 6 per 100 ng of DNA) master mix. Each 4 μl sample requires 1.2 μl of Fugene 6 and 14.8 μl of serum-free DMEM. For identifying the firefly control plasmid linear expression range (**step 1**), add 7.2 μl of Fugene 6 to 88.8 μl of serum-free DMEM in a sterile 1.6 ml microcentrifuge tube (*see* **Note 9**). To identify each target mRNA plasmid linear expression range (**step 2**), add 30 μl of Fugene 6 to 370 μl of serum-free DMEM in a sterile 1.6 ml microcentrifuge tube.
11. Incubate 6× Fugene 6 master mix for 5 min at room temperature and mix gently by pipetting until a homogeneous mixture is obtained.
12. Add 16 μl of Fugene 6 master mix to samples and mix by pipetting.
13. Incubate samples for 15 min to form transfection particles.
14. Remove the 96-well plate with HEK293 cells from the CO_2 incubator and add 5 μl of each sample to the wells, in triplicate (*see* **Note 10**).
15. Mix by swirling the plate gently and place the plate in the CO_2 incubator for 40 h (*see* **Note 11**).

3.6 Dual-Luciferase Assay Readout

1. Allow ONE-Glo EX Luciferase substrate (firefly substrate) and ONE-Glo EX Luciferase buffer from the Nano-Glo Dual-Luciferase Reporter Assay System to reach room temperature. For the assays involving the target mRNA plasmids also allow

NanoDLR Stop and Glo buffer to reach room temperature. Keep NanoDLR substrate (NanoLuc substrate) in the freezer until needed.

2. Add all 10 ml of ONE-Glo EX Luciferase buffer to ONE-Glo EX Luciferase substrate bottle and vortex to mix.
3. Remove 96-well plate(s) with transfected HEK293 cells from the CO_2 incubator.
4. Remove 50 μl of media from each well with a P200 multi-channel pipette, not touching the bottom of the wells, and discard.
5. Add ONE-Glo EX Luciferase substrate mixture to a 25 ml reagent reservoir.
6. Add 50 μl of ONE-Glo EX Luciferase substrate mixture to each well and mix by gentle pipetting. Store remaining ONE-Glo EX Luciferase substrate mixture at −20 °C for future experiments.
7. Protect the 96-well plate from light and incubate it at room temperature for 30 min.
8. Measure luminescence (Fig. 5, Firefly measurement) on a microplate reader using a top reading of 9.5 mm for 0.5 s with a gain of 2500.
9. For firefly control linear expression range experiments, stop here and proceed to Subheading 3.7. For target mRNA (NanoLuc) linear expression range experiments and Effector-TP fusion tethering experiments, proceed to the next steps.
10. Make sufficient NanoLuc substrate master mix for all samples by adding NanoDLR substrate to NanoDLR Stop and Glo buffer to a 15 ml conical tube in a 1:100 ratio. Vortex to mix and add to a reagent reservoir. The NanoLuc substrate mixture should be made fresh for each experiment.
11. Add 50 μl of NanoDLR mixture to each well with a P200 multi-channel pipette and mix by gently pipetting.
12. Protect the 96-well plate from light and incubate it at room temperature for 30 min.
13. Measure luminescence (Fig. 5, NanoLuc measurement) on a microplate reader using a top reading of 9.5 mm for 0.5 s with a gain of 2500.
14. For target mRNA linear expression range experiments, proceed to Subheading 3.8.
15. For Effector-TP tethering experiments, proceed to Subheading 3.10.

3.7 Firefly Control Linear Expression Range Calculations

1. Export firefly luminescence data from the microplate reader.
2. Correct for firefly background signal by subtracting the average signal from all wells with cells but not transfected with plasmids.
3. Average triplicate wells.
4. Plot average firefly signal as a function of firefly control plasmid transfection amount.
5. Select the firefly control transfection amount within the middle of the linear response range. 10 ng of firefly control plasmid was used for the experiments described below.

3.8 Target mRNA (NanoLuc) Linear Expression Range Calculations

1. Export firefly and NanoLuc luminescence data from the microplate reader.
2. Correct for firefly and NanoLuc background signal by subtracting the average signal from all wells with cells but not transfected with plasmids from firefly and NanoLuc measurements, respectively.
3. Normalize background-subtracted NanoLuc signal to background-subtracted firefly signal for each well (Fig. 5, calculation 1).
4. Average triplicate wells (Fig. 5, calculation 2).
5. Plot normalized luminescence as a function of target mRNA plasmid transfection amounts (Fig. 4b).
6. Select the target mRNA plasmid transfection amount within the middle of the linear response range. For experiments described here, 7 ng of target mRNA plasmid was used per well.

3.9 Dual-Luciferase Assay to Evaluate Effector Protein Function as a Function of mRNA Location

1. Plate one 96-well plate with HEK293 cells. Place 100 µl of HEK293 cells at 100,000 cells/ml or 10,000 cells/well (Subheading 3.4).
2. Perform the following steps for all target mRNA plasmids.
3. Prepare master mix with optimized amounts of firefly control, target mRNA, and carrier DNA plasmids for four 20 µl (20 wells) samples. Optimized carrier DNA plasmid concentration is the sum of firefly control, target mRNA, and Effector-TP plasmid concentrations subtracted from 50 ng. For example, here, carrier DNA plasmid transfection concentration per well is:

$$50\ \text{ng}\ (1\ \text{well}) - 10\ \text{ng}\ (\text{firefly control}) - 7\ \text{ng}\ (\text{target mRNA}) - 5\ \text{ng}\ (\text{TP fusions}) = 28\ \text{ng}.$$

A single 20 µl sample (20 wells) will contain 200 ng of firefly control, 140 ng of a target mRNA, and 560 ng of carrier

DNA. Master mix for four 20 μl reactions contains 800 ng of firefly control, 560 ng of a target mRNA, and 2240 ng of carrier DNA.

4. Add appropriate amounts of master mix to three separate 0.2 ml strip PCR tubes, samples 1–3.
5. Add 100 ng of TP, sfGFP, and ZFP36L2-TP plasmids to samples 1–3, respectively (*see* **Note 12**).
6. Bring the total volume of each sample to 20 μl with nuclease-free water.
7. Add 4 μl of each sample to clean 0.2 ml strip PCR tubes, sterilize the outside of tubes with ethanol, and bring samples into the cell culture hood.
8. Prepare 6× Fugene 6:DNA master mix by adding 24 μl of Fugene 6 to 296 μl of serum-free DMEM.
9. Transfect plasmids into a 96-well plate with HEK293 cells (Subheading 3.5, **steps 11–15**). *See* Fig. 5 for the suggested plate layout.
10. Place cells in a CO_2 incubator for 40 h.
11. Perform dual-luciferase assay and measure firefly and NanoLuc luminescence (Subheading 3.6).

3.10 Dual-Luciferase Assay Calculations to Evaluate Effector Protein Function

1. Export firefly and NanoLuc luminescence readings, background-subtract firefly and NanoLuc signals, and normalize all NanoLuc signals by firefly signals per well (Fig. 5, calculation 1).
2. Average normalized NanoLuc signals for triplicate wells (Fig. 5, calculation 2).
3. Calculate fold change of Effector-TP (ZFP36L2-TP was used here) (and sfGFP) sample to TP control sample at each TH position within target mRNA. The calculation involves dividing the (normalized and averaged) NanoLuc signal for the Effector-TP experiments (and sfGFP-TP) by the NanoLuc signal for TP-alone experiments (Fig. 5, calculation 3).
4. Examine the fold change for the no-TH target mRNA wells. Effector-TP transfected samples should be within 10% of TP-alone control samples when performed with the no-TH target mRNA. If not, repeat Subheadings 3.9 and 3.10 with a decreased amount of Effector-TP (and TP) plasmid. If Effector-TP transfected sample is within 10% of the TP-alone sample with the no-TH target mRNA, consider repeating Subheadings 3.9 and 3.10 with an increase in Effector-TP (and TP alone) expression to maximize the effect of the tethered Effector protein.

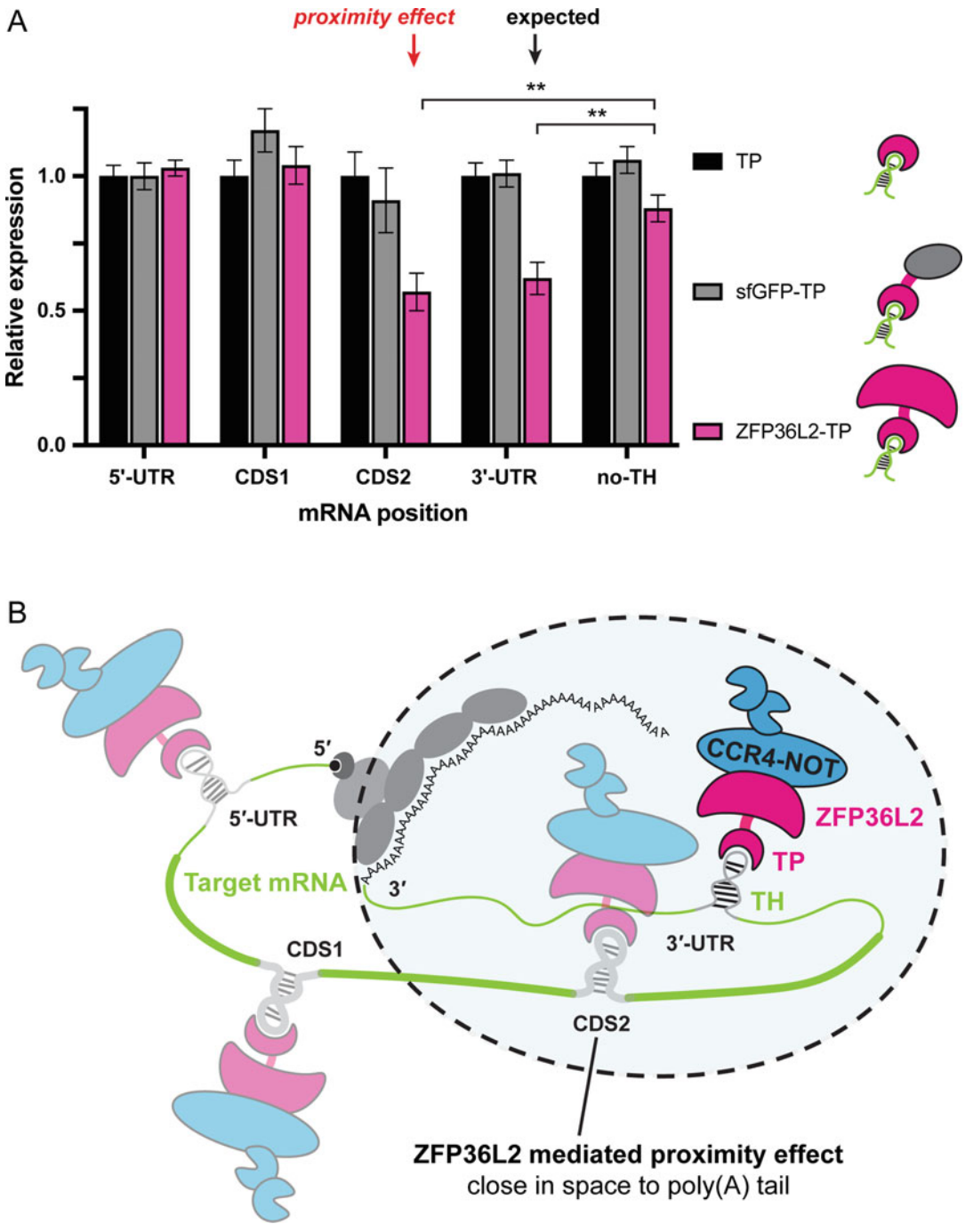

Fig. 6 Proximity effects on ZFP36L2 function. (**a**) Relative expression of target mRNAs as a function of target hairpin (TH) position for fusion proteins linked to the tethering protein (TP) (black), sfGFP-TP (gray), and ZFP36L2-TP (magenta). Means ± SEM are plotted. $^{**}p \leq 0.005$ (two-tailed t-test). (**b**) Scheme illustrating ZFP36L2-mediated proximity effect on target mRNA expression

We used our tethering strategy (Fig. 1) to explore proximity-mediated effects of zinc finger protein 36-like 2 (ZFP36L2), a zinc finger RNA-binding protein that recognizes AU-rich elements (AREs) within the 3'-UTRs of mRNAs. ZFP36L2 acts as an adapter protein that recruits CCR4-NOT, the major deadenylase in mammalian cells [9]. When a ZFP36L2-TP fusion was directed to the TH located in the 3'-UTR of the target mRNA, reporter expression decreased (Fig. 6a). This reduction in expression, based on targeting the 3'-UTR, is consistent with (and expected from) both the known localization of AU-rich elements in 3'-UTRs and with previous studies on zinc finger proteins [10]. Intriguingly, we also observed decreased expression when ZFP36L2 was tethered in the 3′ half of the coding sequence of our target mRNA (CDS2) but not when the protein was tethered in the 5'-UTR or the 5′ half of the coding sequence (CDS1) (Figs. 2a and 6a). ZFP36L2 thus needs only to bind near the poly(A) tail and can act from a site distant from the 3'-UTR via a proximity effect, enabling CCR4-NOT mediated degradation (Fig. 6b).

mRNA regulatory proteins broadly regulate mRNA stability and translation efficiency [1]. The mechanisms by which protein positioning on a given mRNA influence function are poorly understood. Here, we devised an engineered tethering system to address this knowledge gap. We developed a suite of plasmids that allow testing the effect of localizing any Effector protein across an mRNA. The strategy shared here will allow the exploration of proximity-mediated effects for diverse mRNA-binding regulatory proteins on mRNA expression, stability, and translation.

4 Notes

1. Growing plasmids in recombinase deficient strains (recA- or recA mutant) is recommended as using these strains decreases the frequency of multimeric plasmids. Multimerized plasmids have reduced transfection efficiencies but higher expression levels, leading to artifacts in the dual-luciferase assays [11].
2. *E. coli* in the stationary phase is not actively replicating and yields a high proportion of plasmids with supercoiled topology, which have high transfection efficiency and nuclear uptake. Transfection of predominantly supercoiled plasmid DNA is required for optimal dual-luciferase assay results [12].
3. DNA concentration can be calculated from the absorbance at 260 nm. An absorbance ratio (260 nm/280 nm) can be used to assess DNA quality. High absorbance at 280 nm indicates the presence of proteins or phenol. An acceptable 260/280 ratio is in the range of 1.7–2.0, with 1.8 ideal. High absorbance at 230 nm is indicative of the presence of organic compounds. An

acceptable 260/230 range is 2.0–2.2. Using a plasmid preparation with absorbance ratios outside these acceptable ranges can result in poor cellular expression.

4. Dual-luciferase assays can be performed in many different cell lines. Here, we use HEK293 cells as they are transfected with high efficiency and have broad linear expression ranges for both reporters. Note that these differ from HEK293T cells, often used to overexpress recombinant proteins. Plasmids used here contain the SV40 polyadenylation signal, which yields (often excessively) high levels of expression in HEK293T cells.
5. HEK293 cells must be passaged 2 or 3 times after being thawed from −80 °C to yield efficient transfection.
6. HEK293 cells from an over-confluent (>80%) cell culture dish have slower growth rates and lower transfection efficiencies than those grown to (the optimal) 80% confluency. If cells are over-confluent, passage an additional time and then plate cells for the dual-luciferase assay.
7. Trypan blue staining is indicative of a dead cell. Total cell viability above 90% is recommended before plating HEK293 cells for a dual-luciferase assay.
8. Quantification of DNA concentrations using a Qubit instrument often yields lower concentrations than NanoDrop quantification. If DNA concentrations are quantified using a Nanodrop instrument, the total amount of DNA transfected into HEK293 cells may require heuristic correction. We find doubling all individual plasmid amounts and using a Fugene 6: DNA ratio of 3× to work well.
9. Fugene 6 is "sticky" and readily adheres to plastic. Invert the tube several times before preparing the 6× Fugene 6 master mix. Be cautious when adding Fugene 6 to the media. Do not touch the side of the microcentrifuge tube with your pipette. Also, when Fugene 6 and DMEM are combined, the operable volume will be significantly less than expected based on summing the volumes of the two reagents individually. Prepare Fugene 6 and DMEM master mix for 3–5 "extra" samples.
10. Do not use wells on the perimeter of a 96-well plate for samples to be quantified for luminescence (*see* Fig. 5). Corner and edge wells are most prone to evaporation, affecting luciferase assay results [13]. These wells may be used for background luminescence calculations.
11. Steady-state expression occurs 24–48 h after transfection for this assay. Testing a range of time points is important to ensure that NanoLuc does not precipitate or show toxicity during target mRNA expression (*see* Fig. 4a).
12. The optimized transfection concentration for the ZFP36L2-TP fusion in the experiments reported here was 5 ng of plasmid

per well. This low transfection amount for the ZFP36L2-TP plasmid reflects that HEK293 cells are sensitive to exogenous ZFP36L2 expression. A range of transfected DNA concentrations should be tested for other Effector-TP fusions.

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