Development of a Tethered mRNA Amplifier to increase protein expression

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Abstract
Herein, we present a novel method to specifically increase a messenger RNA’s (mRNA) expression at the post-transcriptional level. This is accomplished using what we term a “Tethered mRNA Amplifier.” The Tethered mRNA Amplifier specifically binds an mRNA’s 3′ untranslated region and enhances its stability/translation, often doubling protein output. We test this approach on several transcripts associated with haploinsufficiency disorders and increase their steady-state expression in cell culture. We suggest this approach may be a tenable therapeutic modality with precise activity and broad-spectrum application.

KEYWORDS
gene expression, haploinsufficiency, mRNA metabolism, mRNA therapeutics, mRNA translation

1 | INTRODUCTION

The gene therapy zeitgeist concentrates on correcting or masking DNA mutations at either the DNA or mRNA level.[1] Although these approaches are powerful, they are generally mutation-type specific and lack broad applicability across a patient population. For example, base-editor technology must be tailored to a specific mutation.[2] Antisense oligonucleotide (ASO) technology is dependent on altering pre-mRNA splicing and may not be suitable to address all indications by this gene therapy.[3] Lastly, gene replacement is limited by size constraints. All these limitations are especially problematic in the treatment of haploinsufficiency disorders.

Haploinsufficiency is a genetic condition that arises when a single wild-type allele in combination with a mutant allele is insufficient to produce enough protein, manifesting in disease.[4,5] There are over 300 known human haploinsufficiencies[6] and perhaps thousands more.[7] These have been recalcitrant to disease-modifying technologies because of their etiology, in that precise protein amount is critical for normal physiology. In addition, many haploinsufficiencies arise from spontaneous germ line mutations, for example, in Dravet syndrome.[8] In these cases, a mutation-agnostic approach that enhances gene expression is desirable.

In the treatment of haploinsufficiencies, directly targeting endogenous transcripts may offer a novel therapeutic window. Though changes in gene expression are commonly considered to reflect programmed transcriptional variability, it is a lesser-appreciated fact that extensive regulation of mRNA expression also occurs in the cytosol.[9] Indeed, mRNA stabilization and translation kinetics are key features defining post-transcriptional regulation. The cell achieves post-transcriptional regulation through sequence and/or structural elements that recruit specific positive or negative acting factors to mRNAs. All transcripts are degraded and translated at unique rates, and these rates can be controlled, dramatically impacting per transcript protein output.[10]

Herein, we describe the development of a novel means to enhance the expression of specific mRNAs – we term this a “Tethered mRNA Amplifier.” We document that this technology can be used to control both reporter and endogenous transcripts in human cells. Importantly, we show the broad applicability of the technology, stimulating the translation of transcripts associated with haploinsufficiency disorders.
Lastly, we optimize our mRNA Amplifier such that it can be engineered into current AAV gene therapy strategies. We propose the Tethered mRNA Amplifier approach might provide an efficacious therapeutic modality in the treatment of human haploinsufficiencies.

2 RESULTS AND DISCUSSION

As an entrée toward a disease-modifying technology that could be used to treat haploinsufficiency disorders, we developed a method to specifically enhance an mRNA’s expression. We increase mRNA expression by tethering a known translational stimulator, PABPC1, to the 3'UTR of a target mRNA.\[11,12\] Tethering is achieved by fusing PABPC1 to the RNA binding protein dCas13b and co-expressing a guide RNA (gRNA). The gRNA is critical in that it has anti-sense homology to specific mRNAs and a short hairpin required for dCas13b binding (Figure 1A).\[13\] We demonstrate that this gRNA-targeted tethering enhances both reporter and endogenous mRNAs in a gRNA-dependent manner. First, using HEK293 cells we co-transfect our Tethered mRNA Amplifier alongside a luciferase reporter construct. An approximate 1.5- to 2-fold increase in reporter protein amount is seen when gRNAs directed against the 3'UTR of the luciferase reporter are present. No stimulation occurs when the dCas13b-PABPC1 fusion is expressed alone (Figure 1B; Ctrl). Continuing in HEK293 cells, we stimulatethesetraslation of an endogenous mRNA, MeCP2. An approximate 1.5-fold stimulation of translation is seen using two distinct gRNAs directed against the endogenous MeCP2 transcript's 3'UTR (Figure 1C). The effect of the mRNA Amplifier on MeCP2 expression is dependent on PABPC1 and not seen when just Cas13b is bound (Figure 1D). A mild increase of 15% in MeCP2 transcript steady-state levels is observed when in the presence of the mRNA Amplifier (Figure 1E). These data suggest that the stimulatory role of the Tethered mRNA Amplifier is through both mRNA stability and mRNA translation – known roles for PABPC1 in regulating mRNA metabolism.\[12\]

We further show that the Tethered mRNA Amplifier enhances mRNA expression in multiple cell types; a stimulatory effect on MeCP2 protein expression is seen in SH-SY5Y (a neuronal cell line) and HepG2 (a liver cell line; Figure 1F). Finally, the effect of the Tethered mRNA amplifier appears to be tunable (at least for MeCP2; this remains to be tested for other transcripts) by moving the gRNA to distinct positions within the 3'UTR. In the case of MeCP2, we observed the strongest stimulatory effect as the gRNA is moved closer to the 3' end of the transcript (Figure 1G). This most likely reflects the nature of PABPC1, naturally bound at the transcript's 3' end.

We next tested our Tethered mRNA Amplifier on other transcripts associated with haploinsufficiency disorders. Using SH-SY5Y cells, a model for neurodegenerative disorders, we observe a gRNA-dependent translational stimulation of SYNGAP1\[15\], SHANK3\[16\], CHD2\[17\], and PTEN\[18\] mRNAs (Figure 2A–D). The loss of function of one allele for each of these genes is associated with autism spectrum disorders. In all cases, the stimulatory effect seen was between 1.2- and 2.0-fold for protein expression with an approximately 15%-20% increase in mRNA levels. These data demonstrate that the Tethered mRNA Amplifier is a promising gene therapy candidate for haploinsufficiency and is portable across multiple transcripts of clinical relevance.

Finally, gene therapy vectors such as AAV have payload size limitations of approximately 4.5 kb.\[14\] To minimize the Tethered mRNA Amplifier (5.2 kb), we made specific truncations of PABPC1 and tested their efficacy on MeCP2 expression. PABPC1 contains four RNA-recognition motifs (RRM1–4) at its N-terminus followed by a linker and a Mademoiselle (MLLE) domain at the C-terminus\[12\] (Figure 3A). The RRM domains bind to poly(A) tails while the MLLE domain is known to regulate its stimulatory role in translation.\[12\] Since we are artificially and specifically tethering PABPC1 to mRNAs independent of PABPC1 poly(A)-binding capacity, we reasoned the RRMs would be dispensable for the Tethered mRNA Amplifier’s function. We fused, therefore, just the MLLE domain to dCas13b (3.2 kb). As a first test, we analyzed the putative folding pattern of this new fusion with an in silico approach using AlphaFold v2.0.\[15\] As seen in Figure 3B–E, removal of the RRM domains had minimal impact on the folding of either the MLLE domain or dCas13b itself. In HEK293 cells, we observed that this minimal construct also stimulated MeCP2 mRNA expression in a gRNA-dependent fashion to a similar extent as the full-length construct (Figure 3F). Thus, the Tethered mRNA Amplifier approach is adaptable for use in current clinically efficacious gene therapy vectors.

Here, we have demonstrated a first-of-its-kind approach to enhancing the mRNA transcript expression in human cells. We call this technology a Tethered mRNA Amplifier. We demonstrate that the Tethered mRNA Amplifier can be used in multiple cell types and can enhance mRNA expression of genes associated with haploinsufficiencies, such as SYNGAP1\[15\], SHANK3\[16\], CHD2\[17\], and PTEN\[18\]. The Tethered mRNA Amplifier is tunable by the movement of the tethered moiety to distinct regions within the 3'UTR, imparting informed gene-specific stimulatory effect control. Lastly, we have minimized the mRNA Amplifier to a size that is suitable for gene therapy vehicles such as AAV; although it is feasible to consider delivery of the Tethered mRNA Amplifier through other means such as a lipid nanoparticle.

Further development is needed to demonstrate the utility of a Tethered mRNA Amplifier in the treatment of haploinsufficiencies. Nonetheless, the Tethered mRNA Amplifier has several major advantages over current strategies. First and foremost, the Tethered mRNA Amplifier would be mutation agnostic, amplifying the expression of the normal allele. Mutations associated with haploinsufficiency result in a loss of function for the diseased allele,\[4,5\] and thus amplification of this protein product would likely be inconsequential; this will have to be empirically determined. Second, the Tethered mRNA Amplifier is potentially disease-modifying, correcting the haploinsufficiency by enhancing protein production. Third, the Tethered mRNA Amplifier is broadly applicable; we show that we can specifically enhance the expression of four mRNAs associated with haploinsufficiency and CNS disease. Since PABPC1 regulates most human mRNAs,\[12\] we anticipate that the Tethered mRNA Amplifier could be used to enhance
FIGURE 1  The Tethered mRNA Amplifier approach enhances mRNA expression in multiple cell types (A) schematic representing the Tethered mRNA Amplifier approach. Briefly, PABPC1 is fused to dCas13b. This fusion is recruited to specific mRNAs via a gRNA targeted to the 3′ UTR. In HEK293 cells, the Tethered mRNA Amplifier stimulates gRNA-dependent luciferase reporter (B) and endogenous MeCP2 mRNA (C) expression, using either a luciferase activity assay or western blot, respectively. Red bars in the corresponding schematics represent the approximate positions of two gRNAs used in each experiment. The effect of the Tethered mRNA Amplifier requires PABPC1 (D). The Tethered mRNA Amplifier also enhances MeCP2 mRNA levels (E). A similar stimulatory effect on MeCP2 can also be seen in SH-SY5Y and HepG2 cells (F). (G) The position of the gRNA along the MeCP2 3′ UTR alters the stimulatory effect. (p-values: *<0.05, **<0.005, ***<0.0005). gRNA, guide RNA; mRNA, messenger RNA

almost any human transcript in a gRNA-dependent fashion. In sum, we describe a novel technology that might provide a therapeutic benefit to any one of the nearly 300+ known haploinsufficiency disorders.[6,7] The next steps will be to test the Tethered mRNA Amplifier in a haploinsufficiency model system and determine the efficacy of this approach.

3  |  EXPERIMENTAL SECTION

3.1  |  dCas13b in vitro optimization

dCas13b/PABPC1 construct by cloning the PCR amplified human PABPC1 in pC0054-CMV-dPspCas13b-longlinker-ADAR2DD was
**FIGURE 2** The Tethered mRNA Amplifier enhances the expression of haploinsufficiency disorder-associated transcripts; SYNGAP1 (A), SHANK3 (B), PTEN (C), and CHD2 (D) mRNAs were targeted by the Tethered mRNA Amplifier in a gRNA-dependent manner. The result of protein and mRNA analysis in SH-SY5Y cells are shown. All the protein assays were performed with four distinct biological replicates and at least two biological repeats for the RNA analysis. (p-values: *<0.05, **<0.005, ***<0.0005). gRNA, guide RNA; mRNA, messenger RNA.
FIGURE 3  A minimal Tethered mRNA Amplifier functions in cells (A) schematic of PABPC1 and its functional motifs. (B) AlphaFold predicted model of dCas13b fusion with the MLLE domain of PABPC1 (amino acids 545–636). (C) Predicted model of full-length PABPC1 – all residues except amino acids 545–636 have been hidden post-prediction. (D) Predicted model of full-length dCas13b alone. (E) PyMol alignment of (B–D). (F) Western blots comparing tethering of full-length PABPC1 and MLLE domain alone targeting MeCP2 transcripts in HEK293 cells. Transfected plasmids are indicated above, and protein quantitation is shown below. MLLE, Mademoiselle domain; RRM1–4, RNA recognition motifs.

TABLE 1  List of backbone plasmids and generated construct

| Plasmid number | Description |
|----------------|-------------|
| pJC1204        | PC1-MS2V5-PABPC1 plasmid Addgene#65807 |
| pJC1206        | pC0054-CMV-dPspCas13b-longlinker-ADAR2DD Addgene 103870 |
| pJC1208        | PC0043-Cas3b-crRNA backbone Addgene#103854 |
| pJC1210        | pJC1204 mutated at nucleotide 5606 |
| pJC1211        | pJC1210 digested to remove ADAR2DD and replace with human PABPC1 |
| pJC1246        | dCas13b-MLLE |
| pJC1280        | dCas13b |
| pJC889         | Luciferase-pcDNA3 Addgene #18964 |

by cutting pJC1211 with BamH1/Not1 and re-ligating after blunting the ends.

Multiple sgRNAs targeting the 3′ UTR of the genes of interest were designed using the "nygenome" online tool for the prediction dCas13b guide (Cas13design [nygenome.org])20i (Table 2). These guides were individually cloned into PC0043-Cas3b-crRNA backbone (Addgene#103854)(pJC1208) at BbsI sites. The reaction mix, including forward and reverse oligos in 1XNEB buffer 3.1, was incubated for 5 and 10 min at 95° and 100°, then cooled down at the room temperature for 2 h. Prepared oligos ligated into pJC1208 using T4 DNA ligase (NEB) following the GreenGate protocol.

RNA amplifier technology was tested in HEK293, HepG2, and SH-SY5Y (ATCC CRL 2266) cell lines. HEK293 and HepG2 cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS, and SH-SY5Y cell lines were grown in Eagle’s Minimum Essential Medium (EMEM) with 10% FBS following ATCC guidelines.

Cells were transiently co-transfected with individual sgRNAs along with the dCas13b-PABPC1 fusion plasmid for 48 h using Opti-MEM Reduced Serum Medium (Gibco) and GeneXPlus Transfection Reagent (ATCC ACS-4004). RNA was isolated using the direct-zol RNA Kit (Zymo Research) following the manufacturer’s protocol. cDNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen), and qPCR was performed in Applied Biosystems Real-Time PCR instrument using PowerUp TM SYBR Green master mix (Applied Biosystems) and designed primers (Table 3). The data were analyzed using the CT value compared to a no sgRNA transfection and normalized to ACTB as a housekeeping gene.

Protein was isolated using RIPA buffer, and the western blotting was performed using Mini-protean TGX 4%–15% gels (Bio-Rad). The following antibodies were used for immunoblotting according to the manufacturer’s suggested concentrations; anti-GAPDH (6C5) (Santa Cruz Biotechnology), anti-MeCP2 (D4F3) (Cell Signaling...
### TABLE 2
List of guide RNAs to target the 3’UTR of the genes of interest

| Gene       | Guide RNA          | Sequence                           |
|------------|--------------------|------------------------------------|
| oJC5003    | pJC889-gRNA1       | CACCGCACTATAAGATGGGCGCCTCTAG       |
| oJC5005    | pJC889-gRNA2       | CACCGGAGCTCTAGACATTTAGGTGACAC      |
| oJC5051    | MeCP2-G1           | CACCGAAACACTTTAGGGTTCCGAGCTT       |
| oJC5053    | MeCP2-G2           | CACCGTGCCATTCAAGAAAGAATCCGAGG     |
| oJC5055    | MeCP2-G3           | CACCGATTACACCACATAGTAAGGAAGCCAGG   |
| oJC5057    | MeCP2-G4           | CACCGGAAATTCACAGAAAGGCGCCCCA      |
| oJC5059    | MeCP2-G5           | CACCGACAGAAGATTTGGTGACCCGCA       |
| oJC5061    | MeCP2-G6           | CACCGGCTAAAAATGTATATGCCCCAAAG     |
| oJC5063    | MeCP2-G7           | CACCGCACAAGACAGGTTCAAGAGCCA       |
| oJC5241    | SynGAP-G1          | CACCGCAGAAGATTTAGGGTTCAGGACCCA    |
| oJC5243    | SynGAP-G2          | CACCGGAGAGACAGGTTCAAGAGCCA        |
| oJC5556    | SHANK3-G1          | CACCGGCAACACATAAAGGGCAGGACAG      |
| oJC5558    | SHANK3-G2          | CACCGAAAACCAAATGGAAAGCCACCAA      |
| oJC5560    | PTEN-G1            | CACCGAAGAAAACCTTTAGGCAAGACAGC     |
| oJC5562    | PTEN-G2            | CACCGAGGGAGAATATGATAGGTACGACAG    |
| oJC5564    | CHD2-G1            | CACCGGAATTTGAGGGGTTCAGGAGCCA      |
| oJC5566    | CHD2-G2            | CACCGGAGAAGACAGGTTCAAGAGCCA       |
| oJC5568    | CHD2-G3            | CACCGTAAAGAATCCAAAGGACAGC        |

### TABLE 3
List of the primers used in this study

| Gene       | Primer            | Sequence                           |
|------------|-------------------|------------------------------------|
| oJC4996    | Site mutation oligoA | TCAAGCTGCCAAGGACCGCATCCACAGCGAGAAGTCCAAC |
| oJC4996    | Site mutation oligoB | GCTGGAGCTTCTCGTTGAGTGGGCTTGGCAGTTGA |
| oJC5001    | PABPC1 F          | CAAGAGAAAGAGCGCCCTTTAAG            |
| oJC5001    | PABPC1 R          | CTAAAGGAAGCCGGCCCTTTAAG            |
| oJC5065    | MeCP2-R           | CAGTCCCTTGGAGCTTGGGAGATT           |
| oJC5065    | MeCP2-F           | GTAATTTGATACATCCCCAGGGA            |
| oJC5067    | Actb-F            | CACCTTGGGCAATGAGGGGTTC             |
| oJC5068    | Actb-R            | AGTCTTGTGGGATGTCCAGGT             |
| oJC5240    | MLL-F             | GGATATCGTTCGCAATCCCTCAG            |
| oJC5245    | SynGAP-F          | TCTAGGCCGTATAGAGCAACAGG            |
| oJC5246    | SynGAP-R          | CAGAGACCCCTAGGTCCTCCAG             |
| oJC5546    | SHANK3-F          | AGGATCACACCCGCAGAGATTA            |
| oJC5547    | SHANK3-R          | CTCAGGATCGTGGCCGGAAATC            |
| oJC5548    | PTEN-F            | TCAAGTTCCCTAGGCGGTTACCT            |
| oJC5549    | PTEN-R            | GAGTTTTCTCTGGTCCTGGTTA             |
| oJC5550    | CHD2-F            | CGAAAACAGGACCTGGACCCACT            |
| oJC5551    | CHD2-R            | GATGACGACTGTGTCGACCTGA             |

Technology), anti-CHD2 (cat#4170) (Cell Signaling Technology), anti-PTEN (cat#9552) (Cell Signaling Technology), anti-SynGAP (cat#3200) (Cell Signaling Technology), and anti-pan-Shank, clone N23B/49 (Cat# MABN24) (Millipore).

### 3.2 Luciferase assay
HEK293 cells were transfected with different ratios of pJC889 (Luciferase-pcDNA3 Addgene #18964), pJC1211, and individually two
distinct gRNAs targeting 3′UTR of firefly luciferase transcripts. The optimal ratio (0.2:1:1.5) of plasmid was chosen for this experiment. For luminescence detection, cells were lysed in 100 μL 1X Passive Lysis Buffer (Promega). The lysate was mixed with ONE-Glo EX Reagent (Promega) following the manufacture protocol, and Luminescence was measured using a Lumat LB9507 Luminometer (Berthold Technologies).

### 3.3 | AlphaFold method

Structural predictions of fusion proteins and native Cas13b and PABP were generated using AlphaFold v2.0 as pulled from the GitHub repository https://github.com/deepmind/alphafold from commit “1d43aff941c84dc56311076b58795797e49107b” (ref15). Both native and customized fusion FASTAs were processed according to the AlphaFold documentation using the provided Docker script with the following parameters: “--max_template_date 2020-05-14 – preset = reduced_dbs.” Relaxed predicted structures with the highest pLDDT scores were used for interpretation of the corresponding input FASTAs.

### 3.4 | Quantifications and statistical analysis

All data, shown in figures as bar charts, were quantified as mean ± standard error. Results were considered significant at p < 0.05 as noted throughout figure legends. All experiments, unless otherwise indicated, have been run based on the common practice of at least three biological repeats.

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## CONFLICT OF INTEREST

Jeff Coller is a founder and holds equity in Tevard Biosciences, also holds a patent for the Tethered mRNA Amplifier technology which is licensed between Tevard Biosciences and Johns Hopkins University. These arrangements have been reviewed and approved by Johns Hopkins University following its conflict-of-interest policies.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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