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A Cell-Penetrant Lactam-Stapled Peptide for Targeting eIF4E Protein-Protein Interactions

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Abstract

Eukaryotic translation initiation factor 4E (eIF4E) has emerged as a promising cancer therapeutic target due to its role in the initiation of cap-dependent translation, a process that is accelerated during tumorigenesis. To regulate the initiation of cap-dependent translation, eIF4E participates in protein-protein interactions (PPI) with binding partners, 4E-BP1 and eIF4G, which act as an inhibitor and stimulator of translation, respectively. As both of these proteins interact with eIF4E by utilizing a short, α -helical stretch of amino acids, our laboratory has been working to develop helical mimetics of these proteins, in particular 4E-BP1, to inhibit eIF4E PPIs. Herein, we describe our continued efforts in this area and report the development and characterization of a cell-penetrant lactam stapled peptide for targeting cellular eIF4E.

Graphical Abstract

Keywords

eIF4E; 4E-BP1; stapled peptides; protein-protein interactions

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COMPETING INTERESTS:

The authors declare no competing interests.

SUPPORTING INFORMATION:

Mass spectrometry and HPLC purity spectra for the **Lac-4E-BP1** peptides. Supplemental tables and figures.

1. INTRODUCTION

Eukaryotic translation initiation factor 4E (eIF4E) plays two important roles in the initiation of cap-dependent mRNA translation. eIF4E binds to the m⁷GpppX-cap at the 5' terminus of coding mRNAs to promote the assembly of the eIF4F translation initiation complex, and it promotes the nuclear export of select cap-dependent transcripts.^{1–3} Overexpression of eIF4E induces tumorigenesis, and elevated levels are found in many human cancers, including breast, colorectal, lung, and prostate cancers, glioblastoma and hematological malignancies.^{1, 4–5} Due to the important nature of this initiation factor, it is highly regulated. The primary negative regulator of eIF4E is eIF4E-binding protein 1 (4E-BP1), which sequesters eIF4E from eIF4G, a scaffolding translation initiation factor,⁶ and the eIF4F translation initiation complex by forming an inhibitory protein-protein interaction (PPI) (Figure 1).^{7–9} Interestingly, both 4E-BP1 and eIF4G compete for the same binding site on eIF4E, thus, establishing a competitive binding model for the initiation of cap-dependent translation.⁹ Each uses a disordered peptide region containing the conserved binding motif, YXXXXLϕ (X: variable; ϕ: L, M, F), that undergoes a helical transition upon binding to eIF4E.^{8–9}

Based on the significance of eIF4E in cancer, and the challenges associated with targeting the eIF4E cap-binding site using nucleotide analogues,^{10–14} several laboratories,^{15–18} including our own,^{19–20} have become interested in the development of eIF4E PPI inhibitors. Recently, we reported the development of a hydrocarbon-stapled 4E-BP1 peptide (**HCS-4E-BP1**; Figure 2A), which showed enhanced stabilization of the *i, i+4* helix, nanomolar binding to eIF4E and *in vitro* inhibition of eIF4E PPIs, in addition to promising on-target cellular activity.^{20–21} Unfortunately, this peptide was plagued by poor solubility due to its hydrophobicity making it a non-ideal scaffold for further development. The earliest work in what is known today as peptide stapling came from studies investigating helix stabilization from lactam formation between Lys and Glu or Asp residues.²² Because our sites for alkenyl amino acid incorporation were Lys57 and Glu61 (Figure 2A), we hypothesized that incorporation of a lactam staple between these amino acids may yield a 4E-BP1 stapled peptide with favorable physicochemical properties. Herein we describe our efforts toward this goal and disclose a highly helical and cell-permeable 4E-BP1 lactam-stapled peptide with on-target cellular activity for inhibiting eIF4E PPIs.

2. RESULTS AND DISCUSSION

2.1 Design and *in vitro* characterization of lactam-stapled 4E-BP1 peptides

Although our previous efforts in 4E-BP1 stapled peptide development focused on the 4E-BP1 sequence Gly49–Asn64,²⁰ we were interested in truncating the sequence to improve peptide solubility. As a starting point, we first measured the helicity and activity of shorter linear 4E-BP1 peptides, Arg51–Asn64 and Arg51–Arg63. These studies revealed that little difference existed between these sequences with respect to structural ordering, eIF4E binding affinity or *in vitro* inhibitory activity (Table S1); thus, we chose the shortest 13-amino acid Arg51–Arg63 peptide as our scaffold. Peptide synthesis was carried out as shown in Scheme 1. Macrolactamization of the 4E-BP1 peptide was performed on-resin using acid-labile 4-methyltrityl-protected Lys and 2-phenylisopropyl ester-protected Glu in Fmoc solid-phase peptide synthesis.²³ As a previous report demonstrated equipotent binding

of a wild type 4E-BP1 peptide and one in which Lys57 and Glu61 were swapped (Lys61 and Glu57),²⁴ we synthesized both lactam stapled peptides to investigate the impact of staple asymmetry on helicity, activity and cellular uptake. **LacA-4E-BP1** refers to the wild type sequence, while **LacB-4E-BP1** refers to the Lys/Glu-flipped sequence (Figure 2A).

We first measured the helicities of the lactam-stapled peptides via circular dichroism (CD) spectroscopy. As shown in Figures 2B and 2D, the orientation of the lactam was found to play an important role in promoting peptide helicity, as evidenced by the enhanced structural ordering of **LacB-4E-BP1** (40%) in comparison to **LacA-4E-BP1** and the linear Arg51–Arg63 4E-BP1 sequence (20% for both). This improved helicity translated to both better *in vitro* inhibitory activity, as measured using our PPI catalytic enzyme-linked click chemistry assay (PPI cat-ELCCA),¹⁹ and binding kinetics and affinity, as measured via surface plasmon resonance (SPR) spectroscopy using sensor-immobilized eIF4E (Figures 2C and 2D; sensorgrams can be found in Figure S1). Importantly, the structural, biochemical and biophysical properties of **LacB-4E-BP1** were nearly identical to those of **HCS-4E-BP1**;²⁰ however, the lactam displayed enhanced water solubility and decreased hydrophobicity (Figure S2) as expected. Thus, we were excited to profile this new 4E-BP1 stapled peptide in cells.

Prior to cellular studies, we carried out an orthogonal eIF4E target engagement assay, the cellular thermal shift assay (CETSA).²⁵ CETSA monitors the thermal stabilization of a protein induced upon binding to a ligand. To determine if the **Lac-4E-BP1** peptides were able to engage with eIF4E in the complex cellular milieu, lysate from HEK293 cells was incubated with each peptide and subjected to thermal denaturation. Interestingly, as shown in Figure 3, only **LacB-4E-BP1** was found to promote eIF4E stabilization at the concentrations examined. Although only a ~3-fold difference was observed between the binding affinities of the peptides, we hypothesize that the weaker affinity of **LacA-4E-BP1** is likely enhanced in a non-immobilization-based assay like CETSA leading to negligible eIF4E binding and stabilization. Thus, **LacB-4E-BP1** emerged as a promising stapled peptide candidate for targeting eIF4E PPIs.

2.2 Characterization of cytosolic penetration

While many examples of successful lactam-stapled peptides have been reported for extracellular or membrane-bound targets,^{23, 26} the impact of macrolactamization on cellular uptake has not been extensively studied.^{27–28} Although many methods are available for profiling the total cellular uptake of peptides,²⁹ which may also include signal from endosomally trapped material, we sought a quantitative measurement of cytosolic penetration. Thus, we used the recently reported chloroalkane penetration assay (CAPA) to measure the uptake of the **Lac-4E-BP1** peptides in cells.^{30–31} Briefly, **LacA-** and **LacB-4E-BP1** peptides conjugated at the N-terminus with a chloroalkane tag (ct-peptide) were chemically synthesized. HeLa cells stably expressing HaloTag-GFP anchored to the cytosolic face of the outer mitochondrial membrane³² were pulsed with varying concentrations of ct-peptide, which react with HaloTag upon reaching the cytosol. The pulse step was followed by a subsequent chase step with a chloroalkane-tagged fluorescent dye (ct-dye) to covalently binding to any unreacted HaloTag.³¹ Fluorescence was then measured

via flow cytometry with the intensity being inversely proportional to the penetration of the ct-peptide.³¹

As shown in Figure 4A, surprisingly, **ct-LacB-4E-BP1** was found to be much more cell-penetrant than **ct-LacA-4E-BP1** with CP₅₀ values of $0.25 \pm 0.01 \mu\text{M}$ and $1.5 \pm 0.4 \mu\text{M}$, respectively, where the CP₅₀ refers to the concentration at which 50% normalized fluorescence was observed.³¹ Importantly, the CP₅₀ of **LacB-4E-BP1** was in-line with those measured previously for a cell-penetrant, hydrocarbon-stapled peptide.³¹ As additional comparison between the lactam-stapled peptides, we also compared the percent uptake from our CAPA data at a single concentration of $0.74 \mu\text{M}$. Correlative with the CP₅₀ values, **LacB-4E-BP1** showed significant cellular uptake ($84 \pm 2\%$), while that of **LacA** was much weaker ($20 \pm 9\%$) (Figure 4B). Based on the higher helicity of **LacB-4E-BP1** (Figure 1B), we hypothesize that, despite the lactam staple, this peptide presents a well-organized pattern of hydrophobic surface area sufficient to bind membranes, trigger endocytosis and cause subsequent endosomal escape.^{29, 33–34}

2.3 Characterization of cellular eIF4E PPI inhibition and anti-proliferative activity

To determine the impact of cytosolic penetration on cellular activity, we next characterized the ability of the **Lac-4E-BP1** peptides to inhibit intracellular eIF4E PPIs. To do so, we employed a m⁷GDP cap pull-down assay, which is an affinity assay that can detect eIF4E binding to its protein-binding partners, 4E-BP1 and eIF4G.³⁵ MDA-MB-231 breast cancer cells were treated with **LacA-** or **LacB-4E-BP1** for 6 h, and the resulting cell lysates were analyzed via m⁷GDP affinity chromatography and Western blot. In line with the CETSA and CAPA data, dose-dependent inhibition of eIF4E binding to both eIF4G and 4E-BP1 was observed only with the cell penetrant **LacB-4E-BP1** (Figure 5A). The potency of **LacB-4E-BP1** in this assay was in-line with that of **HCS-4E-BP1**²⁰ in congruence with their comparable *in vitro* biophysical and biochemical properties. Similar, albeit less pronounced dose-dependent effects were also observed in MCF-7 and T47D breast cancer cells treated with **LacB-4E-BP1** (Figures 5B and 5C).

Encouraged by these results, we then measured the effect of the peptides on cell growth. Unexpectedly, unlike **HCS-4E-BP1**,²⁰ no effect on growth was observed following treatment of breast cancer cells with **LacB-4E-BP1** (Figure S3A). Similar results were also found with **LacA-4E-BP1** as expected (Figure S3B). Intrigued by these results, we posited that **LacB-4E-BP1** may suffer from poor stability due to the presence of the lactam staple. Indeed, the half-life of this peptide in mouse plasma was found to be <5 min (Figure S4). To determine if this would translate to cells, MCF-7 cells were treated with peptide for extended times and the lysate was harvested for analysis using the m⁷GDP cap pull-down assay. As shown in Figure S5, the peptide retained activity in this assay for up to 42 h, indicating that stability is not likely playing a role in its lack of growth inhibitory effect. Because our stapled peptides inhibit binding to eIF4E by both its endogenous inhibitor, 4E-BP1, and activator, eIF4G, it is possible that inhibiting both PPIs does not affect growth. Future studies will focus on determining if **HCS-4E-BP1** has additional off-target effects that may be contributing to its anti-proliferative activity, in addition to cellular localization

and expanded phenotypic studies to fully characterize the cellular effects of 4E-BP1 stapled peptide treatment.

3. CONCLUSION

In conclusion, we have synthesized and characterized lactam-stapled peptides based on the eIF4E inhibitory protein, 4E-BP1, for inhibiting eIF4E PPIs. These peptides were designed to enhance water solubility, which was found to be poor in hydrocarbon-stapled peptides previously developed by our group.^{20–21} From these efforts, we identified **LacB-4E-BP1**, which exhibited improved solubility while maintaining *in vitro* inhibitory activity and binding affinity. Importantly, this scaffold was also found to bind to eIF4E via CETSA, exhibit cellular penetrability as measured via CAPA and inhibit eIF4E PPIs in cells. Despite these exciting attributes, the peptide was found to have no effect on cellular viability unlike the hydrocarbon-stapled peptide **HCS-4E-BP1**. This was despite the fact that these stapled peptides both exhibited nearly identical properties, including high helicity, nanomolar binding affinity for eIF4E, cytosolic penetration and intracellular target engagement at concentrations below 1 μ M. Thus, future studies will be focused on detailed comparative studies of **LacB-4E-BP1** and **HCS-4E-BP1**, both structural and biological, in order to identify the differences between these peptides, such as enzymatic stability in cells and unanticipated off-target effects. Such studies will not only enable further development of 4E-BP1 stapled peptides as chemical probes for targeting eIF4E PPIs, but also provide valuable insights for the constrained peptide field as it advances toward the development of next-generation therapeutics.

4. EXPERIMENTAL

4.1. General Materials and Methods:

Linear 4E-BP1 peptides were purchased from New England Peptides and dissolved in H₂O. Fmoc-protected amino acids and Rink amide MBHA resin were purchased from P3 Biosystems and used as received. Fmoc-Glu(2-OiPrPh) was purchased from Sigma and Fmoc-Lys(Mtt)-OH was purchased from ChemImpex; both were used as received. eIF4E and 4E-BP1 proteins were expressed and purified as previously described.^{19–20} IC₅₀ values were determined using PPI cat-ELCCA as reported.¹⁹ Chemiluminescence data was collected on a BioTek Cytation3. eIF4E (9742), 4E-BP1 (9644) and eIF4G (2858) antibodies were purchased from Cell Signaling Technology.

4.2. Data and Statistical Analysis:

All data was analyzed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, www.graphpad.com). Two-sided t-tests were performed using Prism; equal variance between samples being compared was established. Graphs show mean $\pm$ standard deviation as described in the figure legends.

4.3. Lac-4E-BP1 peptide synthesis:

LacA-4E-BP1 and **LacB-4E-BP1** were synthesized on 0.2-mmol scale in a 20-mL fritted syringe using MBHA Rink amide resin (0.2–0.4 mmol/g loading) as according to Scheme

S1. The resin was first swelled with washes of DMF (10 mL), DCM (10 mL) and DMF (10 mL). Fmoc groups were removed by addition of 2× 20% piperidine/90 mM HOBt in NMP (10 mL) and gentle agitation for 10 min at 25 °C, followed by washing as described above. The first amino acid was coupled by addition of Fmoc-Arg(Pbf)-OH (0.3 mmol) pre-activated with HBTU (0.3 mmol) and *N,N*-diisopropylethylamine (DIPEA; 2 mmol) in NMP (5 mL), and agitation for 2–3 h at 25 °C. Acetyl capping was then carried out by addition of acetic anhydride (0.9 mL) and DIPEA (1.6 mL) in NMP (4.5 mL), and agitation for 5 min at 25 °C. Fmoc deprotection and washing then followed. All remaining amino acid couplings were performed by addition of amino acid (1 mmol) pre-activated with HBTU (1 mmol) and DIPEA (2 mmol) in NMP (5 mL), and agitation for 2–3 h at 25 °C, followed by washing and Fmoc deprotection. Reactions were monitored through 10–20 mg resin test cleavages via LC–MS. Macrolactamization was performed following the addition of Fmoc-Lys(Mtt)-OH. The resin was first equilibrated with DCM, and then washed 4× with a solution of 2.25% TFA and 5% TIPS in DCM. The resin was then washed again with DCM, 2% DIPEA in DCM, DMF and DCM. Macrolactamization was subsequently performed using recrystallized PyBOP (6 equiv) and DIPEA (10 equiv) in NMP for 2–3 h at 25 °C. Fmoc SPPS then followed. Upon installation of the final amino acid, the *N*-terminus was acetylated, and the peptide was cleaved from the resin using TFA/phenol/thioanisole/water/TIPS (83/6.25/4.2/4/2/2) for 3 h at 25 °C. For the chloroalkane-tagged (ct) peptides, the *N*-terminus was instead coupled with 4-((2-((6-chlorohexyl)oxy)ethoxy)ethyl)amino)butanoic acid as described.^{30–31} The resulting solution was added to glacial ether (~200 mL) for peptide precipitation. The precipitates were then collected, dissolved, and purified via RP-HPLC. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% HCO₂H in water and B is 0.1% HCO₂H in acetonitrile. Analytical RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a ZORBAX Eclipse SB-C18 column (4.6 × 150 mm; 5 μm) at a flow rate of 1 mL/min, with detection at 214 and 254 nm. Preparative RP-HPLC was performed using an Agilent 1260 Infinity HPLC equipped with a PrepHT SB-C18 column (21.2 × 150 mm; 5 μm) at a flow rate of 18.6 mL/min, with detection at 214 and 254 nm. Fractions containing the desired peptide were confirmed by LC-MS using an Agilent Q-TOF HPLC-MS. Only fractions containing peptide at 95% analytical purity were lyophilized, re-dissolved in 1:1 acetic acid/water, and lyophilized again for use. Stock solutions were dissolved in H₂O. Peptide stock concentrations were determined via amino acid analysis.

4.4. Circular Dichroism (CD) Spectroscopy:

Peptides were dissolved in buffer (5 mM sodium phosphate buffer, pH 7.4) at concentrations between 25–100 μM. CD spectra were recorded on a Jasco I-1500 CD-Spectropolarimeter. Data was collected between 180–260 nm with a step resolution of 0.1-nm and a speed of 50 nm/sec. Five accumulations were taken with the response time set to 1 s, bandwidth to 5 nm, and pathlength of 0.1 cm. The α-helical content of each peptide was calculated by dividing the mean residue ellipticity [φ]₂₂₂obs by the theoretical [φ]₂₂₂ for a helical acetylated peptide of equivalent length.³⁶

4.5. Surface Plasmon Resonance (SPR) Spectroscopy:

SPR was performed using a SensiQ Pioneer instrument and a HisCap chip (three-dimensional hydrogel surface) with 3 channels in series as previously described.²⁰ Channel 1 was activated with NiCl_2 and 10 $\times$ His-eIF4E was immobilized by injection into the running buffer (10 mM HEPES-NaOH, pH 7.5, 225 mM NaCl, 10 mM imidazole, 0.1% Tween-20, and 5% Glycerol). The concentration was adjusted to about 600 RU. 50 μL of peptide was injected at 50 $\mu\text{L}/\text{min}$ over a range of 10 concentrations in duplicate and random order (188 nM–4.0 μM) at 25 °C. The chip was regenerated in between each cycle with an injection 500 mM EDTA followed by reactivation with NiCl_2 and reimmobilization of 10 $\times$ His-eIF4E. The association rate (k_a), dissociation rate (k_d), and binding constant (K_d) were obtained by fitting to a 1:1 interaction model using global data analysis with the Qdat software, which employs the Levenberg-Marquardt non-linear regression algorithm.

4.6. General Cell Culture Methods:

HeLa cells expressing HaloTag-GFP oriented in the cytosol (HGM)³² were grown in DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 $\mu\text{g}/\text{mL}$ puromycin. MDA-MB-231 cells were grown in RPMI1640, 10% FBS, 2 mM L-glutamine. HEK 293 cells were grown in DMEM, 10% FBS and 2 mM L-glutamine. MCF-7 cells were grown in DMEM, 10% FBS, 1 mM sodium pyruvate and 10 $\mu\text{g}/\text{mL}$ of human recombinant insulin. T47D cells were grown in RPMI-1640, 10% FBS, 1 mM sodium pyruvate and 7 $\mu\text{g}/\text{mL}$ of recombinant human insulin. All cells were grown at 37 °C with 5% CO_2 in a humidified incubator. MDA-MB-231 cells were a kind gift from Dr. Nouri Neamati. MCF-7 and T47D cells were a kind gift from Dr. Duxin Sun.

4.7. CETSA:

CETSA was carried out as previously described.²⁵ Cultured HEK 293 cells were trypsinized and washed with PBS. The cells were then diluted in PBS supplemented with protease inhibitors. The cell suspensions were freeze-thawed 3 $\times$ using liquid nitrogen. The soluble fraction (lysate) was separated from the cell debris by centrifugation at 15000 rpm for 25 min at 4 °C. The cell lysates were then divided and treated with either the control (water) or the peptides. After 1 h incubation at room temperature, lysates were divided into 100 μL aliquots and heated at 49 °C and 52.4 °C for 3 min followed by cooling at 25 °C for 3 min in a Peltier gradient thermal cycler. As a control, one set was left at room temperature. The heated lysates were centrifuged 15000 rpm for 25 min at 4 °C in order to separate the soluble fractions from precipitates. The supernatants were transferred to new tubes and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis.

4.8. CAPA:

The chloroalkane penetration assay (CAPA) was performed as described.^{30–31, 37} Briefly, HeLa cells expressing HaloTag-GFP oriented in the cytosol (HGM)³² were seeded in a 96-well plate at a concentration of 35,000 cells per well, to obtain a confluency of 60–80% at the start of the experiment. Growth media was replaced with 100 μL of serum-free Opti-MEM. Serial dilutions of the chloroalkane-labeled peptides were performed in a separate 96-

well plate. 25 μ L of the peptide sample was added to the cells in Opti-MEM and incubated with the cells for 4 h at 37 °C. After the incubation, cells were washed with 50 μ L of fresh Opti-MEM for 15 min. Media was removed, and then cells were incubated with 50 μ L of 5 μ M chloroalkane-labeled tetramethylrhodamine for 15 min. Excess dye was washed away with 50 μ L of fresh Opti-MEM for 30 min. Cells were trypsinized in 40 μ L of 0.05% clear trypsin, resuspended in 180 μ L PBS, and analyzed by flow cytometry. Details about controls, flow cytometry parameters, experimental considerations, and data analysis have been described previously.³⁷

4.9. m⁷GDP cap affinity assay and Western blot:

The cap pull-down assay was carried out as previously described.^{20, 38} Cells were grown in 6-cm dishes and treated with peptides for 6 h. Cells were then lysed in cap pull-down buffer (50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM EDTA, 2 mM DTT and 0.1% Tween 20) containing protease inhibitors. Cell lysate was centrifuged at 15,000 rpm for 25 min. The supernatant was subsequently incubated for 2 h at 4 °C with m⁷GDP-agarose resin. Beads were washed 3 $\times$ with the cap pull-down buffer, 1 $\times$ with TBS and 1 $\times$ with water. Proteins were eluted by boiling in 2 $\times$ LDS sample buffer for 10 min at 70 °C, resolved on a 4–12% Bis-Tris gel, and transferred to PVDF membrane in Towbin's Buffer. The membrane was blocked in 5% milk for 1 h at 25 °C, and then incubated with a primary antibody (overnight at 4 °C) and secondary antibody (1 h at 25 °C). Proteins were visualized by autoradiography. In all cases, the eIF4E level was used for normalization. Figures were formatted in Adobe Illustrator.

4.10. Cell Viability Assay:

The CellTiter-Glo® assay kit was purchased from Promega and was performed according to the manufacturer's instructions. Briefly, 2,000 cells were plated in a white, 96-well tissue culture-treated plate. Cells were treated with the peptides at different concentrations in triplicate and incubated for 6 days. On the sixth day, the cell culture media was replaced with 100 μ L of OptiMEM and then lysed with 100 μ L of CellTiter-Glo® reagent. Total luminescence was read within 1 h using a BioTek Cytation 3 reader. Data was normalized and processed in GraphPad Prism.

4.11. Mouse Plasma Stability Assay:

Peptide (1 μ M final concentration) was incubated with pooled mouse plasma for varying time points from 0 to 60 min. After each time point, an aliquot was taken from the mixture and the reaction was halted by the addition of 4 volumes of cold acetonitrile containing 50 nM of compound CE302 as an internal standard. Samples were centrifuged at 3500 g for 10 min and analyzed using LC-MS/MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Regulation of eIF4E and cap-dependent translation initiation by 4E-BP1 and eIF4G.

Figure 2.
4E-BP1 stapled peptides. (A) Sequences of 4E-BP1, **HCS-4E-BP1** and **Lac-4E-BP1** peptides. (B) Peptide helicities as determined via CD spectroscopy in 5 mM sodium phosphate buffer, pH 7.4, 25 °C. (C) *In vitro* inhibitory activities determined via PPI cat-ELCCA. (D) Tabulated helicity and IC₅₀ values (presented as 95% confidence intervals) and SPR data.

Figure 3. eIF4E target engagement measured by the cellular thermal shift assay (CETSA) in HEK293 cell lysate. eIF4E was detected via Western blot. (A) **LacA-4E-BP1**. (B) **LacB-4E-BP1**.

Figure 4.

Cell penetration of the **Lac-4E-BP1** peptides. (A) CAPA results. (B) Percent HaloTag occupancy following treatment of cells with 0.74 μM peptide. Statistical significance was determined using an unpaired, two-tailed Student's *t* tests (***: $p = 0.0003$).

Figure 5. Inhibition of the eIF4E-4E-BP1 and eIF4E-eIF4G PPIs in cells as determined via the m⁷GDP cap pull-down assay. In all cases, cells were treated with peptides dissolved in water for 6 h and eIF4E was used as a normalization control. (A) MDA-MB-231 cells treated with **LacA-** or **LacB-4E-BP1**. (B), (C) MCF-7 and T47D cells, respectively, treated with **LacB-4E-BP1**.

Scheme 1.

Synthesis of **LacA-4E-BP1**. **LacB-4E-BP1** was synthesized in a similar manner, but the Glu and Lys residues were swapped.