Increased levels of eIF2A inhibit translation by sequestering 40S ribosomal subunits

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ABSTRACT

eIF2A (not to be confused with the alpha subunit of eIF2) was the first initiator tRNA carrier discovered in eukaryotes but its exact function during translation has remained enigmatic. Uncharacteristic of a translation factor, eIF2A resides in the nucleus during normal physiological conditions and is shuttled to the cytoplasm during cell stress. eIF2A knockout mice show normal protein synthesis but have shortened lifespans and develop metabolic syndromes in adulthood. Attempts to study eIF2A *in vitro* have been limited by the inability to achieve high yield of soluble recombinant protein. Here, we developed an optimized recombinant expression and purification paradigm that produces ~360X more soluble protein than previous reports to study the function of human eIF2A. We first supplemented mammalian *in vitro* translation reactions with recombinant human eIF2A and found that eIF2A inhibits translation of multiple reporter mRNAs. Using sucrose gradient ultracentrifugation along with various translation inhibitors to capture and measure the levels of translation complexes at different stages, we identified that eIF2A inhibits translation prior to start codon recognition. eIF2A also inhibited translation directed by all four types of cap-independent viral IRESs, including those that do not require ribosomal scanning, initiation factors, or initiator tRNA, suggesting excess eIF2A sequesters the 40S subunit. Importantly, reactions supplemented with additional 40S subunits rescued translation and pull-down assays show evidence of direct binding between recombinant eIF2A and purified 40S subunits. These data support a model that eIF2A must be kept away from the translation machinery to avoid sequestering the 40S ribosomal subunit.

Running title: eIF2A-mediated translational inhibition

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INTRODUCTION

Canonical mRNA translation initiation uses the heterotrimeric eukaryotic initiation factor 2 (eIF2) bound to GTP to deliver the initiator tRNA (Met-tRNA$_i^{Met}$) to the P site of the 40S ribosomal subunit; ultimately forming the ternary complex (TC; eIF2•GTP•Met-tRNA$_i^{Met}$). The TC along with eIF1, eIF1A, eIF3, eIF5, and the 40S subunit form the 43S pre-initiation complex (PIC) that is recruited to the 5' m^{7}G cap bound by eIF4F.[1] The PIC then scans 5'-to-3' in search of an AUG start codon.[2, 3] Once the initiator tRNA in the P site base pairs with the AUG start codon, eIF2 hydrolyzes GTP and releases the initiator tRNA, stimulating the dissociation of itself and most eIFs. The 60S subunit then joins, which is aided by eIF5B, to form the 80S ribosome.[4] While eIF2 is the dominant Met-tRNA$_i^{Met}$ carrier in the cell, several other factors have been reported to bind and deliver Met-tRNA$_i^{Met}$ to the 40S subunit, namely eIF2A, eIF2D, and MCT-1•DENR (Multiple Copies in T-cell Lymphoma 1 and Density Regulated Protein complex).[5-7] Multiple reports suggest eIF2D and MCT-1•DENR function in translation re-initiation[6-8], but the exact role of eIF2A remains unknown (for an in-depth review on eIF2A history see Komar and Merrick, 2020).[9]

eIF2A is a 65 kDa monomeric protein (non-homologous to eIF2) that was discovered nearly 50 years ago and was originally purified from rabbit reticulocyte lysate.[5, 10] In 1975, Adams *et al*. used methionyl-puromycin synthesis assays to demonstrate that eIF2A was able to deliver Met-tRNA$_i^{Met}$ to the 40S subunit to form a functional 80S ribosome; however, eIF2A was much less efficient of a Met-tRNA$_i^{Met}$ carrier than eIF2 for endogenous mRNA.[11] While eIF2A was first thought to be the functional ortholog of prokaryotic IF2 due to them both being monomeric and requiring a mRNA template to deliver Met-tRNA$_i^{Met}$, it soon became known that eIF2 (and not eIF2A) was the primary Met-tRNA$_i^{Met}$ carrier in eukaryotes. Intriguingly, endogenous eIF2A protein is as or even more abundant than the eIF2 subunits (α, β, γ)[12] (**Figure 1A** and **Table S1A**) and has a similar reported binding affinity for Met-tRNA$_i^{Met}$ as eIF2, (12.4 nM and 15.0 nM, respectively).[13, 14] However, little is known on a transcriptome wide level which mRNAs or open reading frames require eIF2A. CLIP-seq-based experiments to identify eIF2A-bound transcripts are lacking. To our knowledge, only a single published report has used ribosome profiling of eIF2A knockout (KO) cells which show a decrease in the ratio of uORF (many of which were non-AUG encoded) to ORF translation during cell stress.[15]

Atypical of translation factors, Kim *et al*. demonstrated that eIF2A is primarily restricted to the nucleus during normal growth conditions but shuttles to the cytoplasm during cell stress in Huh7 cells.[13] Other reports have provided evidence that eIF2A may selectively function in translation of specific mRNAs or open reading frames. Sendoel *et al*. demonstrated that eIF2A knockdown (KD) decreases the translation of the UUG-encoded upstream open reading frame (uORF) in *Binding immunoglobin protein* (*BiP*) during cell stress.[15] Starck *et al*. conclude that Leu-tRNA^{Leu} can be used to initiate translation at CUG codons, which is not inhibited by NSC119893 that suppresses canonical eIF2-mediated initiation.[16] Additionally, knockdown of eIF2A decreased translation of CUG-encoded transcripts.[16] The authors speculated that eIF2A also uses Leu-tRNA^{Leu} for initiation[16]; however, direct binding between eIF2A and Leu-tRNA^{Leu} was not confirmed. Additionally, Liang *et al*. reported an isoform of *PTEN (PTEN-α)*, a gene commonly mutated in cancer, is translated from a CUG-encoded uORF during cell stress and knockdown of eIF2A leads to decreased *PTEN-α* translation.[17] eIF2A may also regulate IRES-mediated translation in yeast and human cells[13, 18-20], although this is not entirely clear as conflicting results focusing on hepatitis C virus (HCV) and sindbis virus IRESs and eIF2A have been reported.[21, 22] In yeast, eIF2A-null cells have unchanged polysome profiles compared to control strains; yet genetic and physical interactions with eIF5B and eIF4E supports that eIF2A functions in translation.[18, 23] eIF2A KO mice developed a metabolic syndrome and had

decreased life spans by one year of age; however, this seems to be independent of translation levels as protein synthesis appeared to be unchanged.[24]

Purifying eIF2A has historically been challenging and has slowed mechanistic interrogation of its function. The initial purification scheme for eIF2A from rabbit reticulocyte lysate included eight steps and produced a seemingly homogenous final product but did not yield protein that was consistently active that could deliver labeled Met-$tRNA_i^{Met}$ to 40S subunits.[10] Merrick and Anderson identified a contaminating factor (later identified and named eIF2D) that was also able to bind and deliver Met-$tRNA_i^{Met}$ to 40S subunits.[6] Due to the high similarity in chromatographic properties between eIF2A and eIF2D, a new purification scheme was developed that selected for eIF2A but, for unknown reasons, led to inactive eIF2A.[6, 10] Kim *et al*. reported the ability to produce 10 µg of recombinant human eIF2A from 6 L of *E. coli* cultures which had low nM affinity for initiator tRNA.[13]

Here, we developed a robust method for production of recombinant human eIF2A from *E. coli* with ~360-fold higher yield. Titrating recombinant eIF2A into mammalian *in vitro* translation reactions inhibited translation of multiple reporter mRNAs and reduced formation of 80S ribosomes and 48S initiation complexes, suggesting eIF2A inhibits translation prior to start codon recognition. eIF2A also inhibited translation directed by all four types of cap-independent viral IRESs, including those that do not require ribosomal scanning, initiation factors, or initiator tRNA, suggesting increased eIF2A sequesters the 40S subunit. Reactions supplemented with additional 40S subunits rescued translation and pull-down assays show evidence of direct binding between recombinant eIF2A and purified 40S subunits. These data support a model that eIF2A must be kept away from the translation machinery to avoid sequestering the 40S ribosomal subunit.

RESULTS

Recombinant eIF2A inhibits AUG- and near-cognate-initiated translation *in vitro*

Native eIF2A can be purified to a relatively mixed level of purity from rabbit retic lysate but can be expressed recombinantly in *E. coli* and purified to high purity. However, overexpression of eIF2A in prokaryotic systems usually requires large volumes for little yield (i.e., 6 L of culture yields 10 µg soluble protein).[13] To overcome these barriers, we adapted an autoinduction expression system using Rosetta 2 (DE3) cells and dual-tagged human eIF2A to purify only full-length MBP-eIF2A-His6 protein (**Figure 1B**). By comparison, a single 50 mL autoinduction culture yielded 31.2 µg (an ~ 360-fold increase in yield/mL culture). We consistently observed a sub-stoichiometric amount of GroEL chaperone (identified by mass spectrometry) co-eluting with recombinant eIF2A. Compared to the His6-MBP tag alone, MBP-eIF2A-His6 did not overexpress well but was retained in the soluble fraction. Switching MBP for GST or SUMO did not increase expression; however, fusing NusA to eIF2A allowed very large amounts of insoluble NusA-eIF2A-His6 to accumulate (data not shown).

To first assess eIF2A function during translation, we titrated recombinant MBP-eIF2A-His6 into mammalian *in vitro* translation reactions using rabbit reticulocyte lysate (RRL) programed with nanoLuciferase (nLuc) reporter mRNA. His6-MBP control protein was largely inert in the system (IC_{50} = 14.38 µM with 7.97-40.34 µM 95% CI), but, to our surprise, recombinant eIF2A robustly inhibited translation (IC_{50} = 0.19 µM with 0.14-0.25 µM 95% CI) (**Figure 1C**). We do not believe the fused MBP tag contributes to inhibition as cleaving off the MBP tag with TEV protease still renders recombinant eIF2A inhibitory (**Figure 1D-E**). To ensure this noted inhibition was not due to human eIF2A being expressed in *E. coli* and folding improperly (despite being soluble), we expressed and purified eIF2A from insect Sf9 cells using a baculovirus system (**Figure 1F**), which yielded 507 µg from a single 50 mL pellet (an ~ 6000-

fold increase in yield/mL culture) and repeated the titration in mammalian *in vitro* translation reactions programed with nLuc reporter mRNA. Insect cell produced recombinant human eIF2A robustly inhibited translation (IC_{50} = 0.37 μM with 0.27-0.50 95% CI) (**Figure 1G**), demonstrating that recombinant eIF2A derived from *E. coli* and insect cells act similarly to inhibit translation.

Several reports have used biochemical and genetic approaches to conclude that eIF2A is able to initiate translation at near-cognate start codons, at times even with Leu-$tRNA^{Leu}$.[15-17] Additionally, altered levels of eIFs have been noted to decrease start codon fidelity to favor near-cognate start codons.[25] Thus, we next asked if eIF2A also inhibited translation that initiated at near-cognate CUG and GUG start codons. We generated AUG-, CUG-, GUG-, and AAA-encoded 3XFLAG-Renilla Luciferase (RLuc) reporters (**Figure 2A**) that only differed by the start codon and tested how eIF2A affected translation of each. Importantly, the CUG and GUG mutations reduced translation of RLuc by ~2-fold and the AAA mutant (which does not support initiation) nearly eliminated RLuc translation (**Figure 2B**), demonstrating our reporters are specific for each start codon. Conflicting the reported positive role of eIF2A in near-cognate-mediated translation initiation, eIF2A equally inhibited translation of AUG-, CUG-, and GUG-encoded RLuc mRNA (**Figure 2C-D**). Together, these data reveal a new inhibitory phenotype caused by eIF2A.

Distinct regions of the N- and C-termini are required for eIF2A-mediated translational repression

To better understand how eIF2A inhibits translation, we next sought to determine which elements of eIF2A are required for inhibition. Empirically determined structures for mammalian eIF2A are not available, but AlphaFold predicts a globular nine bladed β-propeller at the N-terminus and three alpha helices at the C-terminus connected by flexible linkers (**Figure 3A, left**).[26, 27] Kim, E. *et al*. previously reported that eIF2A has three separate domains to bind Met-$tRNA_i^{Met}$, eIF5B, and mRNA (**Figure 3A, right**).[28] Using these defined regions and the AlphaFold predicted structure, we generated a large series of eIF2A deletion mutants (**Figure 3B** and **Figure S2A**) and tested how each inhibited translation of nLuc mRNA (**Figure 3C** and **Figure S2B**). Of our 13 mutants, only seven—1-415, 1-430, 1-437, 1-471, 1-480, 416-585, and 533-585—purified to a respectable level of purity with sub-stochiometric levels of GroEL chaperone (**Figure 3B**). However, all seven of these mutants did not inhibit translation (**Figure 3C**). Of the six that did not purify well (**Figure S1A**), only 1-503, 1-529, and 1-556 demonstrated some translation inhibition (**Figure S1B**). From these data, we designed two new mutants that harbored the complete N-terminus (1-437), a GGS linker, and a portion of the C-terminus (either 504-556 or 529-556) (**Figure 3D**) that purified with low levels of GroEL and inhibited translation at least two-fold. These data support that a single domain in eIF2A is not responsible for translation inhibition.

eIF2A inhibits translation prior to 48S initiation complex formation independent of initiation factors or initiator tRNA

To decipher which step of translation is being inhibited by eIF2A, we used sucrose gradient ultracentrifugation along with various translation inhibitors to capture and measure the levels of translation complexes at different stages on nLuc reporter mRNA. Cycloheximide (CHX) is an elongation inhibitor that binds the E-site of 60S subunits and blocks 80S translocation. When added prior to the start of translation, CHX will capture ribosomes near the start codon of nLuc reporter mRNA after one elongation cycle as CHX requires a deacylated tRNA in the E site to arrest 80S ribosomes. Using this strategy, we observed that eIF2A decreased 80S formation ~2-fold (**Figure 4A-B, S2A**). To determine if eIF2A inhibits translation before 80S formation, we repeated the sucrose gradient experiments and trapped 48S initiation complexes (the 43S pre-initiation complex bound to mRNA at start codons) after start codon

recognition but before 60S subunit joining by adding the non-hydrolysable GTP analog, GMPPNP. If eIF2A inhibited translation after start codon recognition, we would hypothesize unchanged levels of 48S complexes. However, our data shows a 3-4-fold decrease in 48S complex levels (**Figure 4C-D, S2A**). These data support that eIF2A inhibits translation prior to start codon recognition.

To inhibit translation prior to start codon recognition, eIF2A could target initiation factors, the 40S subunit, 43S pre-initiation complex formation and recruitment, or 43S scanning. To decipher among these possibilities, we took advantage of the ability to direct translation using various combinations of initiation factors via viral internal ribosome entry sites (IRESs). Specifically, we used the prototypical type I, II, III, and IV IRESs from poliovirus (PV), encephalomyocarditis virus (EMCV), hepatitis C virus (HCV), and cricket paralysis virus (CrPV IGR), respectively (**Figure 5A**).[29] Importantly, IRES reporters were A-capped and harbored a strong hairpin in the 5' untranslated region (UTR) to prevent canonical translation. Upon testing translation reactions programmed with each IRES reporter, we observed that eIF2A inhibited translation independent of the subset of eIFs used and did not require 43S scanning (**Figure 5B**). Translation directed from CrPV IGR IRES which supports initiation independent of any initiation factors, the initiator tRNA, or scanning was also notably inhibited, suggesting that eIF2A either targets the 40S or 60S subunit (**Figure 5B**).

Given that 60S subunit joining is downstream from 48S formation and eIF2A decreases 48S levels (**Figure 4C-D**), we hypothesized that eIF2A is sequestering the 40S subunit, resulting in the ability of eIF2A to inhibit translation independent of eIFs or initiator tRNA (**Figure 5A-B**). To test this idea, we supplemented translation reactions with excess purified 40S subunits to rescue translation. In agreement with our hypothesis, excess 40S subunits severely blunted the ability of eIF2A to inhibit translation (**Figure 5C-D**). Together, these data suggest that eIF2A is sequestering the 40S ribosome and inhibiting translation.

eIF2A directly binds the 40S subunit

Previous work has shown that eIF2A co-sediments with 40S and 80S ribosomes in HEK293FT and yeast cells during cell stress[23, 28], but there is limited evidence of eIF2A directly binding to the 40S ribosome. Initial work using filter binding assays identified eIF2A could deliver Met-tRNA$_i^{Met}$ to 40S subunits with an AUG trinucleotide with 4 mM Mg^{2+}.[5] To determine whether eIF2A interacts with 40S subunits in our translation reactions, we took advantage of the C-terminal His6 tag present on recombinant eIF2A for pulldown assays with Ni^{2+}-NTA magnetic beads and then probed by Western blot for RPS6 (es6) as a marker for 40S subunits. Indeed, RPS6 did co-purify, in a formaldehyde-dependent manner, with MBP-eIF2A-His6 but not with the negative control His6-MBP (**Figure 6A**). The dependence on formaldehyde, a zero-distance crosslinker, suggests a transient but specific association. To test whether eIF2A directly interacts with 40S subunits, we repeated the His6-pulldowns with purified 40S subunits. Again, RPS6 was co-purified with MBP-eIF2A-His6 but not His6-MBP (**Figure 6B**). In total, these data support a model in which eIF2A directly binds and sequesters the 40S subunit to inhibit translation initiation (**Figure 7**).

DISCUSSION

A correct balance of initiation factors is required for tight control over translation initiation and start codon recognition. For example, overexpression of eIF1 and eIF5 increases and decreases the stringency of start codon selection in cells, respectively.[25] Here, we show that increased levels of eIF2A inhibit translation initiation independent of eIFs and the initiator tRNA by sequestering the 40S subunit. This raises the question—how do cells prevent eIF2A from inhibiting translation? Kim *et al*. has reported that in Huh7 cells, eIF2A primarily localizes to the

nucleus during typical normal growing conditions but re-localizes to the cytoplasm during cell stress[13], possibly to then aid in stress-specific translation. Restraining eIF2A to the nucleus during conditions of high canonical translation could be a robust method for cells to restrict eIF2A from inhibiting translation and sequestering 40S subunits. Whether eIF2A in other cell types share similar localization dynamics has not been reported to our knowledge but would be of value to determine the molecular switch that governs eIF2A re-localization. Nuclear-to-cytoplasmic re-localization during cell stress has been noted for other post-transcriptional regulatory mechanisms and are typically regulated by reversible phosphorylation.[30-32] Future work will be required to determine whether eIF2A is reversibly post-translationally modified and regulated in a similar manner during cell stress.

Previous work reported that residues 462-501 of eIF2A interact with eIF5B[28], the GTPase that controls subunit joining after start codon recognition. It is possible that recombinant eIF2A is sequestering eIF5B and subsequently inhibiting a late step of initiation. However, this is likely not the main mechanism of inhibition for many reasons. First, inhibiting eIF5B should have no effect on 48S initiation complex levels when captured by GMPNPP, but eIF2A-mediated inhibition clearly reduced initiation complex levels (**Figure 6C-D**). Second, eIF2A-mediated inhibition was severely blunted when translation reactions were supplemented with extra 40S subunits, suggesting 40S subunits are being targeted by eIF2A (**Figure 5D**). Third, translation initiation directed by the CrPV IGR IRES, which does not require eIF5B, was also inhibited by eIF2A (**Figure 5B**). Lastly, deletion of the residues in eIF2A that were mapped to interact with eIF5B did not prevent inhibition (**Figure S1B**).

After exploring the Alphafold predicted structure of human eIF2A[26, 27] and the recent cryo-EM structure of the human 48S initiation complex[3], we noticed the eIF2A N-terminal domain and the central domain in eIF3b both form a nine-bladed β-propeller. In eIF3b, this propeller directly contacts RPS9e (uS4) and eIF3i.[33, 34] While our experiments did not test if eIF2A displaces eIF3b or other eIF3b subunits from 40S subunits, we do not conclude that this possibility as the primary mode of inhibition due to the fact that the CrPV IGR IRES was susceptible to eIF2A (**Figure 5B**). Additionally, multiple eIF2A mutants harboring just the N-terminal domain alone (1-415, 1-430, and 1-437) did not inhibit translation (**Figure 3C**).

Instead, our mutational analysis testing different eIF2A domains reveals that a single domain on its own is not able to inhibit translation (**Figure 3**). Initial work described eIF2A as being able to deliver initiator tRNA to the 40S subunit only when an AUG trinucleotide was present[5, 10, 11], raising the possibility that eIF2A delivered initiator tRNA to the P site during start codon recognition. However, the exact positioning of eIF2A on the 40S with or without initiator tRNA is still unknown. Future structural work investigating an eIF2A•40S complex will be critical to further define how eIF2A contributes to start codon recognition. A major hurdle of recovering a high yield of recombinant eIF2A has now been lifted as we demonstrate here both bacterial- and insect cell-synthesized human eIF2A are active and recombinant eIF2A interacts with 40S subunits independent of any additional factors.

MATERIALS AND METHODS

Plasmids

Full-length human eIF2A (Ref seq RNA # NM_032025.5) was synthesized by IDT and was cloned in pET His6 MBP TEV LIC cloning vector (1M), which was a gift from Scott Gradia (Addgene plasmid # 29656), through ligation-independent cloning (LIC) using Novagen's LIC-qualified T4 DNA polymerase (Sigma # 70099-M) as described by Q3 Macrolab (http://qb3.berkeley.edu/macrolab/). The His6-tag was deleted from the N-terminus and inserted

at the C-terminus. Deletions were achieved using the Q5 Site-Directed Mutagenesis Kit (NEB # E0552S. For recombinant expression and purification from insect cells, MBP-eIF2A-His6 was PCR amplified to incorporate a C-terminal FLAG tag and then subcloned into pFastBac1(Thermo Fisher # 10359016).

pcDNA3.1(+)/nLuc-3XFLAG was previously described.[35] pcDNA3.1(+)/3XF-RLuc (with AUG, CUG, GUG, AAA start codon variants) was constructed by subcloning RLuc from pRL-SV40 (which was a kind gift from Aaron Goldstrohm; Promega # E2231) via PCR amplification to insert the N-terminal 3XFLAG tag. IRES-containing nLuc reporters were generated using an overlapping PCR method and cloned into pcDNA3.1(+) or pcDNA3-1D. The PV IRES template was pcDNA3 RLUC POLIRES FLUC and was a gift from Nahum Sonenberg (Addgene plasmid # 45642). The EMCV IRES and HCV IRES templates were kind gifts from Aaron Goldstrohm. pcDNA3.1-D/CrPV IGR IRES nLuc-3XFLAG was previously described[35] but was additionally modified to contain a strong hairpin upstream of IRES element to block scanning pre-initiation complexes. All IRES reporters contained the same strong hairpin upstream of the IRES element (which is noted in the complete reporter sequence in the Supplemental Information). Hairpin insertion (for IRES reporters) and all mutations were introduced using the Q5 Site-Directed Mutagenesis Kit (NEB # E0554S).

All plasmids were propagated in TOP10 E. coli (Thermo Fisher # C404006), purified using the PureYield Plasmid Miniprep or Midiprep Systems (Promega # A1222 and A2495), and validated by Sanger sequencing at The Ohio State University Comprehensive Cancer Center Genomics Shared Resource (OSUCCC GSR). Nucleotide sequences of all reporters and full-length recombinant proteins are provided in the Supplemental Information.

Recombinant protein expression and purification

Bacteria derived recombinant His6-MBP and MBP-eIF2A-His6 were produced in Rosetta 2(DE3) *E. coli* (Sigma # 71397-4) using MagicMedia *E. coli* Expression Medium (Thermo Fisher # K6803) supplemented with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol for auto-induction. A 5 mL starter culture in LB media supplemented with 50 µg/mL kanamycin, 35 µg/mL chloramphenicol, and 1% glucose (w/v) was inoculated with a single colony and grown overnight at 37°C, 250 rpm. 1 mL of a fresh overnight starter culture was then used to inoculate 50 mL of room temperature MagicMedia and incubated for 48 hrs at 18°C, 160 rpm in a 250 mL baffled flask. After auto-induction, cultures were pelleted and stored at -20°C for purification later. Recombinant proteins were purified using a dual affinity approach, first using the C-terminal His6-tag, then the N-terminal MBP-tag. Cell pellets were resuspended and lysed with BugBuster Master Mix (Sigma # 71456) using the recommended 5 mL per 1 g wet cell pellet ratio for 10 min at room temperature with gentle end-over-end rotation (10-15 rpm). Lysates were placed on ice and kept cold moving forward. Lysates were cleared by centrifugation for 20 min at 18,000 rcf in a chilled centrifuge (4°C). Lysates were then incubated with HisPur Cobalt Resin (Thermo Fisher # 89965) in a Peirce centrifugation column (Thermo # 89897) for 30 min at 4°C with gentle end-over-end rotation. Columns were centrifuged in a pre-chilled (4°C) Eppendorf 5810R for 2 min at 700 rcf to eliminate the flow through and then were washed 5X with two resin-bed volumes of ice-cold Cobalt IMAC Wash Buffer (50 mM Na_3PO_4, 300 mM NaCl, 10 mM imidazole; pH 7.4) in a pre-chilled (4°C) Eppendorf 5810R for 2 min at 700 rcf. His-tagged proteins were then eluted in a single elution step with two resin-bed volumes of ice-cold Cobalt IMAC Elution Buffer (50 mM Na_3PO_4, 300 mM NaCl, 150 mM imidazole; pH 7.4) by gravity flow. Eluates were then incubated with Amylose resin (NEB # E8021) in a centrifugation column for 2 hrs at 4°C with gentle end-over-end rotation. Columns were washed 5X with at least two bed-volumes of ice-cold MBP Wash Buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA; pH 7.4) by gravity flow. MBP-tagged proteins were then eluted by a single elution step

with two resin-bed volumes of ice-cold MBP Elution Buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose; pH 7.4) by gravity flow. Recombinant proteins were then desalted and buffer exchanged into Protein Storage Buffer (25 mM Tris-HCl, 125 mM KCl, 10% glycerol; pH 7.4) using a 7K MWCO Zeba Spin Desalting Column (Thermo Fisher # 89892) and, if needed, concentrated using 10K MWCO Amicon Ultra-4 (EMD Millipore # UFC803024). Recombinant protein concentration was determined by Pierce Detergent Compatible Bradford Assay Kit (Thermo Fisher # 23246) with BSA standards diluted in Protein Storage Buffer before aliquoting in single use volumes, snap freezing in liquid nitrogen, and storage at -80°C.

Insect derived recombinant His6-mEGFP-FLAG and MBP-eIF2A-His6-FLAG was expressed in ExpiSf9 cells using the ExpiSf Expression System Starter Kit (Thermo Fisher # A38841) following the manufacture's protocol. Bacmids were produced by transforming MAX Efficiency DH10Bac Competent Cells and selecting for integrated transformants (white colony) on LB Agar supplemented with 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 300 µg/mL Bluo-gal, 40 µg/mL IPTG. Single integrated transformants were then re-streaked on selective LB Agar and then used to inoculate 100 mL of LB supplemented with 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline with overnight incubation at 37°C, 250 rpm. Bacmids were isolated from 30 mL of culture using PureLink HiPure Plasmid Midiprep Kit (Thermo Fisher # K210004). 25 mL of ExpiSf9 cells at 2.5 x 10^6 cells/mL in ExpiSf CD medium in a 125 mL non-baffled vented PETG flask (Thermo Fisher # 4115-0125) were transfected with 12.5 µg of bacmid using 30 µL ExpiFectamine Sf Transfection Reagent in 1 mL Opti-MEM I Reduced Serum Media and incubated at 27°C, 195 rpm. P0 baculovirus stocks were collected after 5 days and stored at 4°C for less than a week before long-term storage at -80°C. For protein expression, 240 mL of ExpiSf9 cells at 5 x 10^6 cells/mL in ExpiSf CD medium in a 1L non-baffled vented PETG flask (Thermo Fisher # 4115-1000) were treated with 800 µL ExpiSf Enhancer and incubated at 27°C, 195 rpm for 22 hr. 3 mL of P0 baculovirius stock was then added and allowed to incubate for 72 hr at 27°C, 195 rpm. Cells were then harvested (50 mL culture pellets), snap frozen in liquid nitrogen, and stored at -80°C. A single cell pellet from 50 mL of culture was then lysed in 16 mL (4 pellet volumes) of ice-cold Lysis Buffer (25 mM Tris, 300 mM KCl, 10% glycerol (v/v), 0.5% Igepal CA-630 (v/v), protease inhibitor EDTA-free (ThermoFisher # A32955), phosphatase inhibitor cocktail (ThermoFisher # A32957); pH 7.5) with end-over-end rotation for 15 min at room-temperature. Lysates were cleared by centrifugation at 214,743 rcf at 4°C in S55A rotor using a Sorvall Discovery M120SE centrifuge and added to 1 mL of Pierce Anti-DYKDDDDK Affinity Resin (Thermo Fisher # A36803) in Peirce centrifugation column (Thermo # 89897) for 2 hrs at 4°C with gentle end-over-end rotation. Columns were washed 3X with at least two-bed volumes of ice-cold Lysis Buffer and once with room-temperature Elution Buffer without Peptide (25 mM Tris, 125 mM KCl, 10% glycerol (v/v); pH 7.5) by gravity flow. FLAG-tagged proteins were then eluted with room-temperature Elution Buffer with 2.5 mg/mL 3XFLAG Peptide (Thermo # A36806) for 15 min at room-temperature. Eluates were placed on ice and then incubated with Amylose resin (NEB # E8021) in a centrifugation column for 2 hrs at 4°C with gentle end-over-end rotation. Columns were washed 5X with at least two bed-volumes of ice-cold MBP Wash Buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA; pH 7.4) by gravity flow. MBP-tagged proteins were then eluted by a single elution step with two resin-bed volumes of ice-cold MBP Elution Buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose; pH 7.4) by gravity flow. Recombinant proteins were then desalted and buffer exchanged into Protein Storage Buffer (25 mM Tris-HCl, 125 mM KCl, 10% glycerol; pH 7.4) using a 7K MWCO Zeba Spin Desalting Column (Thermo Fisher # 89892). Recombinant protein concentration was determined by Pierce Detergent Compatible Bradford Assay Kit (Thermo Fisher # 23246) with BSA standards diluted in Protein Storage Buffer before aliquoting in single use volumes, snap freezing in liquid nitrogen, and storage at -80°C.

In vitro transcription

nLuc-3XFLAG and 3XFLAG-RLuc plasmids were linearized with PspOMI. PV IRES nLuc, EMCV IRES nLuc, HCV IRES nLuc, CrPV IGR IRES nLuc plasmids were linearized with XbaI. Digested plasmids were purified using the DNA Clean and Conentrator-25 (Zymo Research # 11-305C). 0.5 µg of linearized plasmid was used as template in a 10 µL reaction using the HiScribe T7 High Yield RNA Synthesis Kit (NEB # E2040S) with an 8:1 ratio of cap analog to GTP, producing ~90% capped RNA, for 2 h at 30 °C. Template DNA was digested with the addition of 1 µL RNase-free DnaseI (NEB # M0303S; enzyme stock at 2,000 U/mL) for 15 min at 37 °C. mRNAs were subsequently polyadenylated using E. coli poly(A) polymerase (NEB # M0276S) with the addition of 5 µL 10X buffer, 5 µL10mM ATP, 1 µL E. coli poly(A) polymerase (enzyme stock at 5,000 U/mL), and 28 µL Rnase-free water for 1 hr at 37 °C. mRNAs were purified using RNA Clean and Concentrator-25 (Zymo Research # 11-353B), eluted in 50 µL Rnase-free water, aliquoted in single-use volumes, and stored at -80°C. nLuc-3XFLAG and 3XFLAG-RLuc mRNAs were co-transcriptionally capped with the 3´-O-Me-m7G(5')ppp(5')G RNA Cap Structure Analog (NEB # S1411S). All viral IRES nLuc mRNAs were co-transcriptionally capped with the A(5')ppp(5')G RNA Cap Structure Analog (NEB # S1406S).

In vitro translation and luciferase assays

10 µL *in vitro* nLuc mRNA translation reactions were performed in the dynamic linear range using 3 nM mRNA in the Flexi Rabbit Reticulocyte Lysate (RRL) System (Promega # L4540) with final concentrations of reagents at 20% RRL, 10 mM amino acid mix minus Leucine, 10 mM amino acid mix minus Methionine,100 mM KCl, 0.5 mM MgOAc, 8 U murine Rnase inhibitor (NEB # M0314L), and 0-3.4 µM recombinant protein. Reactions were performed for 30 min at 30°C and then terminated by incubation on ice. nLuc luciferase signal was measured by mixing 25 µL of Nano-Glo Luciferase Assay System (prepared 1:50 as recommended; Promega # N1120) with 25 µL of diluted reactions (diluted 1:5 in Glo Lysis Buffer; Promega # E2661) and incubated at room temperature for 5 min, and then read on a Promega GloMax Discover multimode plate reader. RLuc mRNA translation reactions were performed identically as described above but were incubated at 30°C for 90 min (this allowed the weaker RLuc enzyme to give signal above background for the start codon mutants) and mixed diluted lysates with an equal volume of Renilla-Glo Luciferase Assay System (prepared 1:100 as recommended; Promega # E2710) for 10 min at room temperature.

Western blotting

10 µL translation reactions were performed as described above, then mixed with 40 µL of 2X reducing LDS sample buffer (Bio-Rad # 1610747) and heated at 70°C for 15 min. 15 µL was then separated by standard Tris-Glycine SDS-PAGE (Thermo # XP04200BOX) and transferred on to 0.2 µm PVDF membrane (Thermo # 88520). Membranes were then blocked with 5% (w/v) non-fat dry milk in TBST (1X Tris-buffered saline with 0.1% (v/v) Tween 20) for 30 min at room temperature before overnight incubation with primary antibodies in TBST at 4°C. After three 10 min washes with TBST, membranes were incubated with HRP-conjugated secondary antibody in TBST for 1 hr at room temperature and then washed again with three 10 min washes with TBST. Chemiluminescence was performed with SuperSignal West Pico PLUS (Thermo # 34577) imaged using an Azure Sapphire Biomolecular Imager. Rabbit anti-GAPDH was used at 1:1,000 (Cell Signaling # 5174S). Rabbit anti-RPS6 (Cell Signaling # 2217S) was used at 1:1,000. Mouse anti-FLAG was used at 1:1,000 (Sigma # F1804). HRP-conjugated goat anti-rabbit IgG (H+L) (Thermo # 31460) and HRP-conjugated goat anti-mouse (Thermo # 31430) were both used at 1:10,000.

Sucrose gradient ultracentrifugation, RNA extraction, and RT-qPCR.

In vitro translation reactions were programmed as described above with 3 nM nLuc mRNA (final) except were scaled up to 100 µL, contained 2 µM recombinant protein (final), and used untreated RRL (Green Hectares). Reactions were spiked with 5 mM GMPPNP (stock at 100 mM in 100 mM Tris-HCl, pH 7.7; Sigma # G0635-5MG) and 100 µg/mL cycloheximide (stock at 100 mg/mL in DMSO; Sigma # C1988) or 100 µg/mL cycloheximide only and 48S initiation complexes and 80S ribosomes were allowed to form for 10 min at 30°C, respectively. Reactions with GMPPNP were supplemented with additional Mg^{2+} (1 mM final) to balance the cation sequestered by the added GMPPNP (this was optimized by determining 48S abundance with control reactions in sucrose gradients). As a negative control, an mRNA only sample of 100 µL 3 nM reporter mRNA in Rnase-free water was used. Reactions were then placed on ice, diluted with 100 µL of ice-cold Polysome Dilution Buffer (20 mM Tris-HCl, 140 mM KCl, 5 mM $MgCl_2$; pH 7.5) and layered on top of a linear 5-30% (w/v) buffered sucrose gradient (10 mM Tris-HCl, 140 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 µg/mL cycloheximide; pH 7.4) in a 14 mm × 89 mm thin-wall Ultra-Clear tube (Beckman # 344059) that was formed using a Biocomp Gradient Master. Gradients were centrifuged at 35K rpm for 3 hrs at 4°C in a SW-41Ti rotor (Beckman) with maximum acceleration and no brake using a Beckman Optima L-90 Ultracentrifuge. Gradients were subsequently fractionated into 0.5 mL volumes using a Biocomp piston fractionator with a TRIAX flow cell (Biocomp) recording a continuous $A_{260\ nm}$ trace. Total RNA was extracted from 400 µL of each fraction (spiked with 0.2 ng exogenous control FFLuc mRNA; Promega # L4561) by adding 600 µL TRIzol (Thermo Fisher # 15596018) and following the manufacturer's protocol. Glycogen (Thermo Fisher # R0561) was added at the isopropanol precipitation step. The resulting RNA pellet was resuspended in 30 µL nuclease-free water. 16 µL of extracted RNA was converted to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad # 1708841). cDNA reactions were then diluted 10-fold with nuclease-free water and stored at -20°C or used immediately. RT-qPCR was performed in 15 µL reactions using iTaq Universal SYBR Green Supermix (Bio-Rad # 1725124) in a Bio-Rad CFX Connect Real-Time PCR Detection System with 1.5 µL diluted cDNA and 250 nM (final concentration) primers. For each fraction, nLuc reporter mRNA abundance was normalized to the spiked-in control FFLuc mRNA using the Bio-Rad CFX Maestro software (ΔΔCt method). Abundance of total signal in each fraction was calculated using $Q_n = 2^{\Delta\Delta Ct}$ and $P = 100 \times Q_n/Q_{total}$ as previously described.[36] Primers for RT-qPCR can be found in **Table S1**.

40S ribosomal subunit purification

250 mL of native rabbit reticulocyte lysate (Green Hectares) was thawed overnight at 4ºC, transferred to 25 x 89 mm Ultra-Clear thin-wall tubes (Beckman # 344058) (typically 38 mL per tube) and centrifuged at 28,000 rpm (140,992.2 rcf) for 4 hours in a SW28 rotor using a Beckman Coulter Optima L-90K Ultracentrifuge. The supernatant aspirated and each crude ribosome pellet was resuspended in 500 µL ice-cold Buffer A (20 mM HEPES, 500 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose; pH 7.5) by gentle orbital shaking overnight at 4°C in the dark and then gentle manual pipetting. Resuspensions were combined in a 1.7 mL microcentrifuge tube, gently mixed using end-over-end rotation (12 rpm) for 30 min at 4°C, and centrifuged at 18,000 rcf for 10 min at 4°C. Supernatants were pooled and puromycin was added to a final concentration of 5 mM, incubated 15 min on ice, followed by gentle mixing for 15 min at 37ºC to separate subunits and then snap frozen in liquid nitrogen and stored at -80ºC. Puromycin-treated crude ribosomes were thawed on ice, diluted 1:4 with ice-cold Buffer B (20 mM HEPES, 500 mM KCl, 1 mM $MgCl_2$, 2 mM DTT; pH 7.5) and 400 µL was loaded onto a 5-30% (w/v) buffered sucrose gradient (in Buffer B) in a 14 mm × 89 mm thin-wall Ultra-Clear tube (Beckman # 344059) that was formed using a Biocomp Gradient Master. Six gradients were centrifuged at 35K rpm for 3 hrs at 4°C in a Beckman SW-41Ti rotor with maximum acceleration and no brake using a Beckman Optima L-90 Ultracentrifuge. Gradients were subsequently fractionated into 0.5 mL volumes using a Biocomp piston fractionator with a TRIAX flow cell (Biocomp) recording

a continuous $A_{260\ nm}$ trace. Fractions that contained the leading edge of the 40S peak were pooled and pelleted in a 11 x 34 mm thin-wall polycarbonate tube (Thermo # 45315) at 55,000 rpm for 18 hrs at 4°C in a Sorvall S55-S rotor using a Sorvall Discovery M120 SE Micro-Ultracentrifuge. The supernatant was removed and each pellet was resuspended in 25 µL ice-cold Buffer C (20 mM HEPES, 100 mM KCl, 2.5 mM $MgCl_2$, 0.25 M sucrose, 2 mM DTT; pH 7.5) and pooled. A_{260} values were measured using a Nanodrop spectrophotometer and concentration was calculated using 1 A_{260} = 65 pmol/mL.[37] Purified 40S subunits were aliquoted into 5 µL volumes, flash frozen in liquid nitrogen, and stored at -80ºC.

His6 pulldown binding experiments

In 20 µL, 3 µg recombinant His6-MBP or MBP-eIF2A-His6 was mixed with 20% RRL in 100 mM KCl, 10 mM amino acid mix minus methionine, 10 mM amino acid mix minus leucine, and 0.5 mM MgOAc with 3 nM nLuc mRNA (same as *in vitro* translation) or with 0.9 µM purified 40S subunits in Binding Buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 2 mM DTT; pH 7.5) and were incubated at 25ºC for 10 min. Indicated samples were then crosslinked with 0.25% (v/v) formaldehyde (Sigma # F79-500) for 30 min at 25ºC and quenched with 20 µL 100 mM Tris-HCl, pH 7.5. Samples were diluted 1:10 in ice-cold Wash Buffer (20 mM Tris-HCl, 140 mM KCl, 10 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 10 mM imidazole; pH 7.5) and added to 20 µL HisPur Ni-NTA Magnetic Beads (Thermo #888831) and incubated 30 min at 4ºC with end-over-end rotation. For 40S subunit binding experiments, HisPur Ni-NTA Magnetic Beads were blocked with 1 µg/µL BSA (Invitrogen # AM2616) and 2 µg/mL yeast tRNA (ThermoFisher # AM7119) in Wash Buffer for 1 hr at 4ºC with end-over-end rotation. Beads were then washed 5X with 400 µL ice-cold Wash Buffer. Bound proteins were then eluted with 200 µL 2X reducing LDS sample buffer (BioRad # 1610747) and heating for 15 min at 70ºC. 20 µL of eluate was analyzed by SDS-PAGE and Western blotting as described above.

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FIGURES

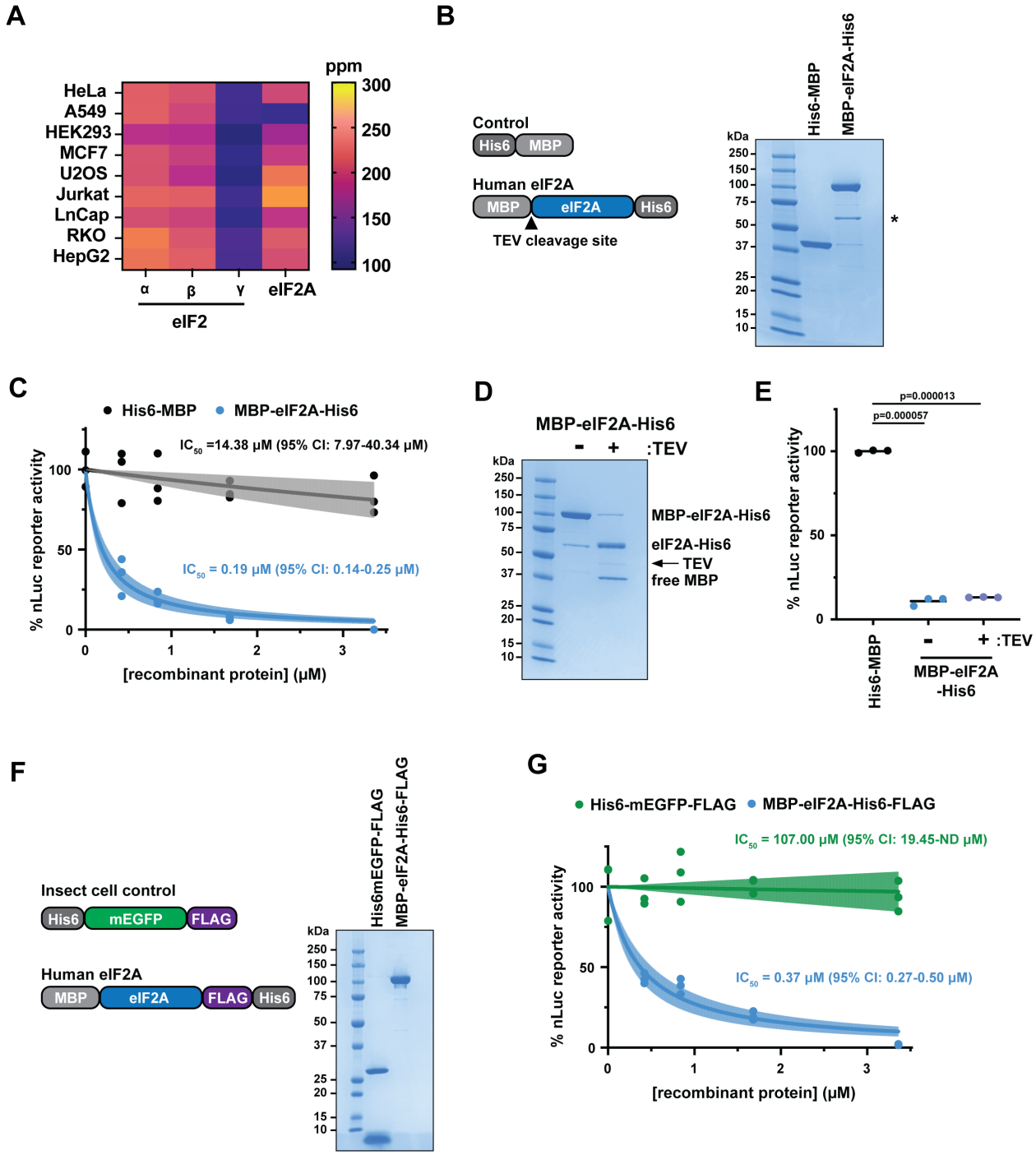


Figure 1. Recombinant eIF2A inhibits translation *in vitro*. A) Comparison of endogenous eIF2(α, β, γ) and eIF2A protein levels in various human cell types. Data was obtained from pax-db.org using the Geiger, MCP, 2012 dataset. ppm = parts per million. B) SDS-PAGE and Coomassie stain of recombinant His6-MBP and MBP-eIF2A-His6. 2 µg of protein was loaded. * = co-purified bacterial Gro-EL chaperone. C) *In vitro* translation of nLuc mRNA with a titration (0-3.4 µM) of the indicated recombinant proteins. IC_{50} values were determined for the His6-MBP

tag (14.38 μM with 7.97-40.3 μM 95% CI) and MBP-eIF2A-His6 (0.19 μM with 0.14-0.25 μM 95% CI). n=3 biological replicates. A non-linear regression was used to calculate the IC_{50} and is shown as the line with the 95% confidence interval (CI) included as a watermark. D) SDS-PAGE and Coomassie stain of MBP-eIF2A-His6 with and without TEV protease treatment. E) Comparison of nLuc mRNA translation inhibition in RRL with uncleaved and TEV cleaved MBP-eIF2A-His6 (1.68 μM). Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction. F) SDS-PAGE and Coomassie stain of His6-mEGFP-FLAG and MBP-eIF2A-His6-FLAG. 2 μg of protein was loaded. G) In vitro translation of nLuc mRNA with a titration (0-3.4 μM) of recombinant protein. IC_{50} values were determined for His6-mEGFP-FLAG (107.2 μM with 19.45-ND μM 95% CI) and MBP-eIF2A-His6-FLAG (0.37 μM with 0.27-0.50 μM 95% CI). n=3 biological replicates. A non-linear regression was used to calculate the IC_{50} and is shown as the line with the 95% CI included as a watermark. CI = confidence interval. ND = not determined.

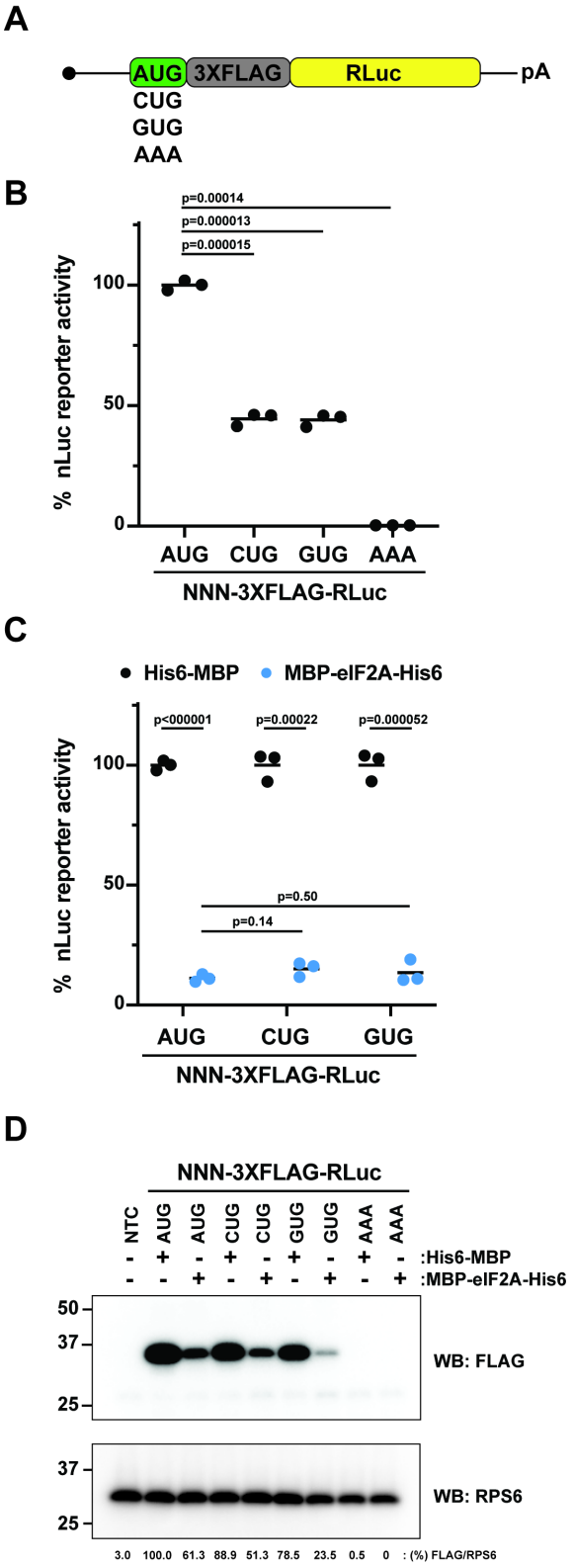


Figure 2. eIF2A inhibits AUG- and near-cognate-initiated translation. A) Diagram of Renilla Luciferase (RLuc) reporter mRNAs harboring various start codons and a N-terminal 3XFLAG tag. B) Comparison of AUG- and non-AUG-3XFLAG-RLuc reporter mRNAs translated *in vitro.*

Luciferase levels are normalized to AUG-3XFLAG-RLuc. Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction. C) Response of *in vitro* translation reactions programmed with AUG- and non-AUG-3XFLAG-RLuc reporter mRNAs in the presence of 1.68 μM His6-MBP or 1.68 μM MBP-eIF2A-His6. Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction. D) Anti-FLAG Western blot comparisons eIF2A-mediated inhibition (1.68 μM) of 3XFLAG-RLuc reporters in RRL. RPS6 was used as a loading control. FLAG signal was normalized to RPS6. NTC = No Template Control.

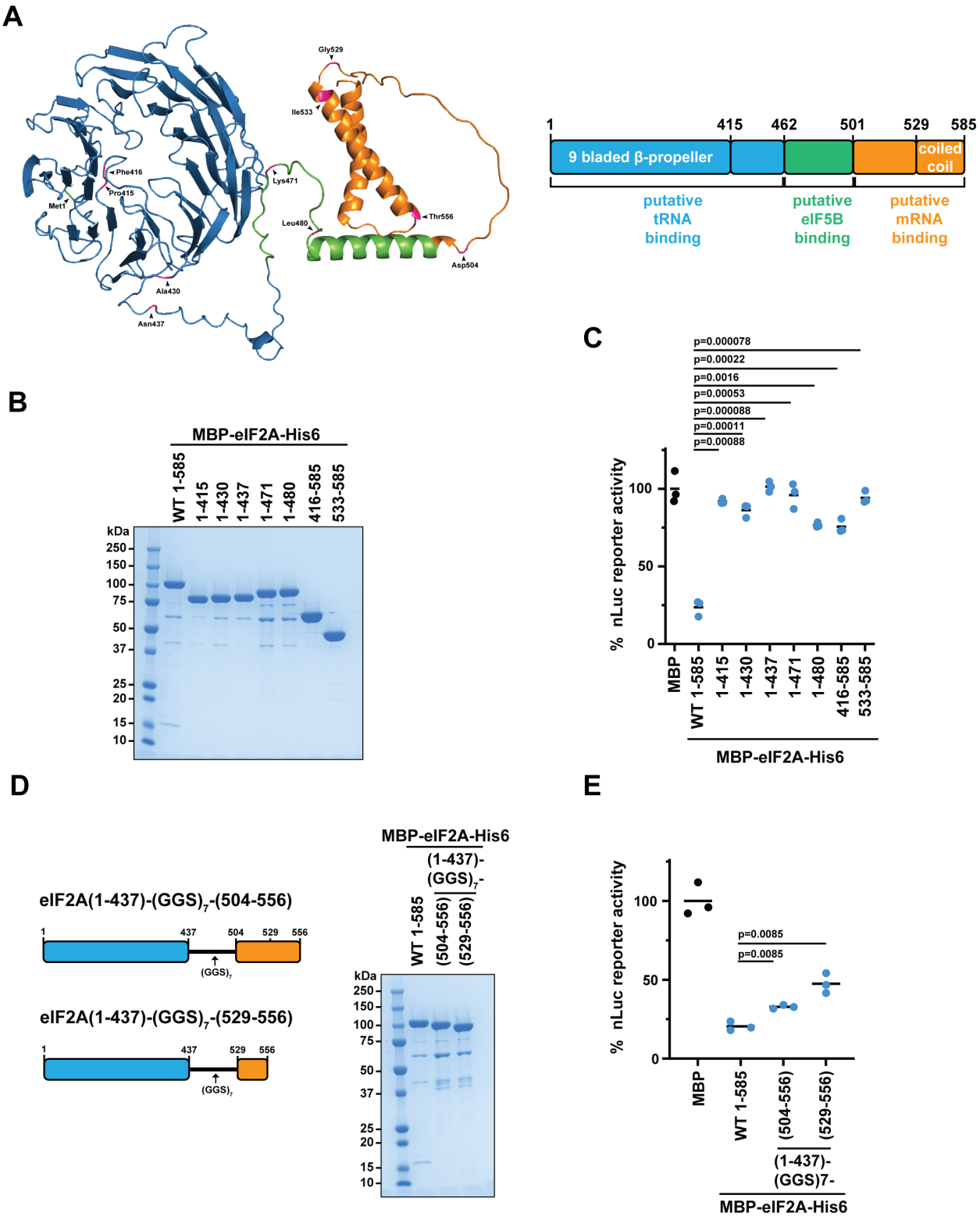


Figure 3. The eIF2A N- and C-termini alone do not inhibit translation. A) AlphaFold structural prediction of human eIF2A with mutation sites from this study colored in pink (left). Schematic of the three previously annotated eIF2A domains with any structural motifs labeled

(right). B) SDS-PAGE and Coomassie stain of recombinant WT and mutant MBP-eIF2A-His6. 2 µg of protein loaded. C) Response of RRL translation reactions programmed with 3XFLAG-RLuc containing 1.68 µM recombinant His6-MBP or MBP-eIF2A-His6. Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction. D) SDS-PAGE and Coomassie stain of recombinant dual N- and C-terminal MBP-eIF2A-His6 mutants. 2 µg of protein loaded. E) Response of RRL translation reactions programed with 3XFLAG-containing 1.68 µM recombinant His6-MBP or MBP-eIF2A-His6. Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction.

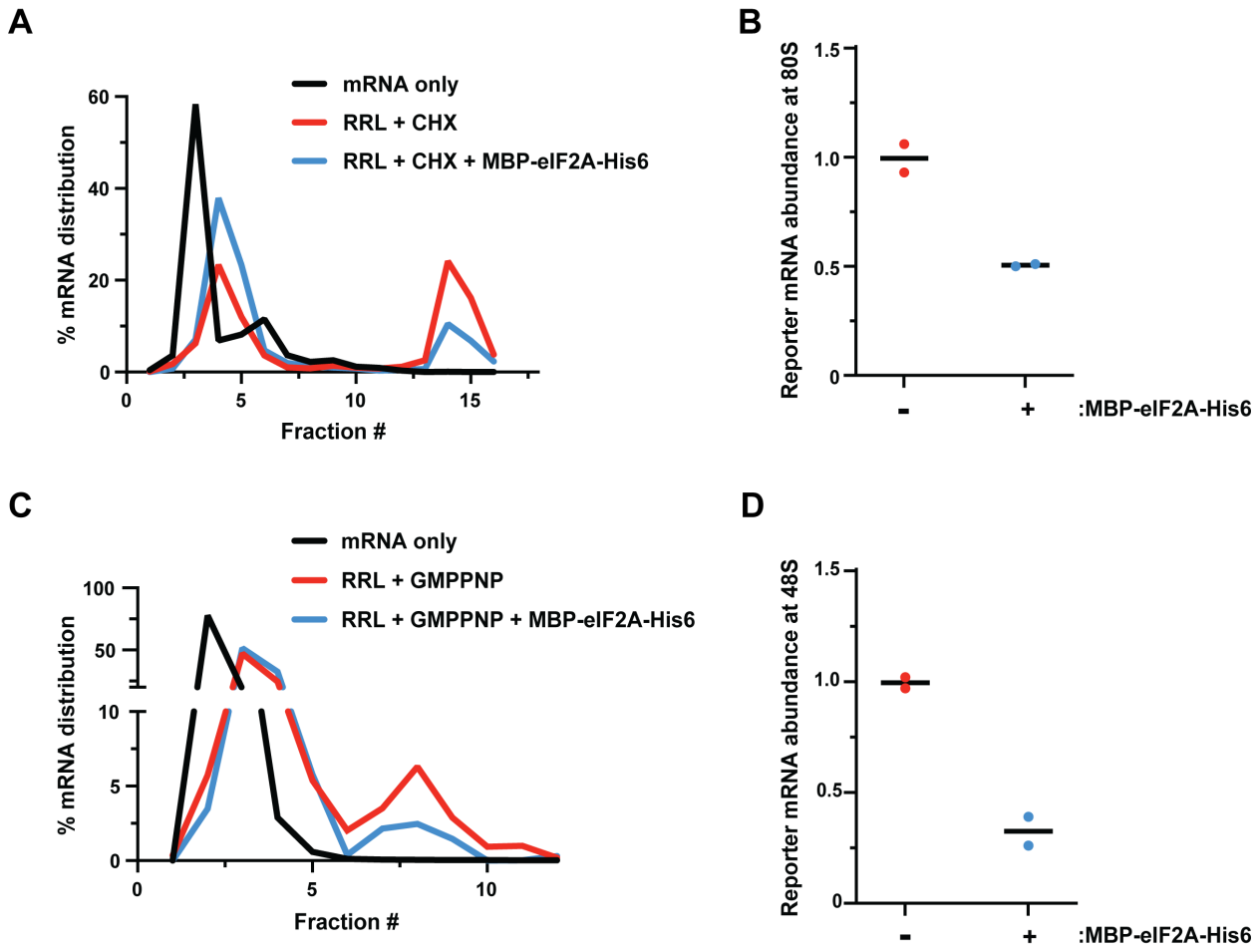


Figure 4. eIF2A inhibits translation prior to 48S initiation complex formation. A) nLuc mRNA distribution along a 5-30% (w/v) sucrose gradient supplemented with 100 µg/mL cycloheximide (CHX) to stall 80S ribosomes after one elongation cycle and with either His6-MBP or MBP-eIF2A-His6 (2.0 µM). B) Quantification of mRNA abundance at 80S peak from A. Bars represent the mean. n=2 biological replicates. C) Same as in A, but additionally supplemented with 5 mM GMPPNP to capture 48S initiation complexes at the start codon. D) Quantification of mRNA abundance at 48S initiation complex peak from C. Bars represent the mean. n=2 biological replicates.

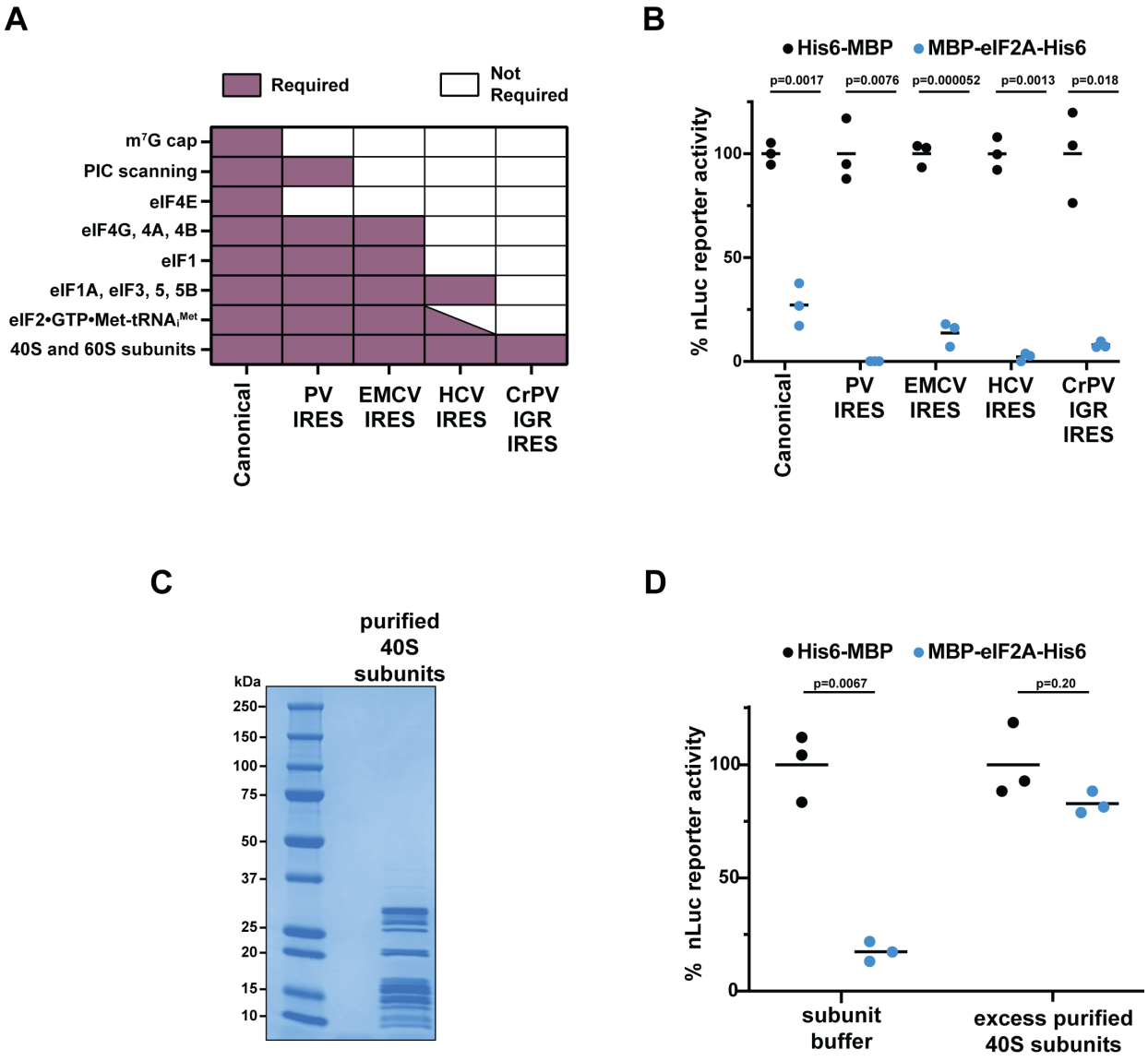


Figure 5. eIF2A inhibits translation independent of eIFs and initiator tRNA. A) Requirements for translation initiation of IRESs types I-IV, which are represented by poliovirus (PV), encephalomyocarditis virus (EMCV), hepatitis C virus (HCV), and cricket paralysis virus intergenic region (CrPV IGR), respectively. B) Response of canonical- and IRES-driven nLuc mRNA *in vitro* translation with either His6-MBP or MBP-eIF2A-His6 (1.68 μM). Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction. C) SDS-PAGE and Coomassie stain of purified 40S ribosomal subunits from RRL. D) Response of eIF2A-mediated inhibition of *in vitro* nLuc mRNA translation with subunit buffer or excess 40S ribosomal subunits (0.27 μM final). Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction.

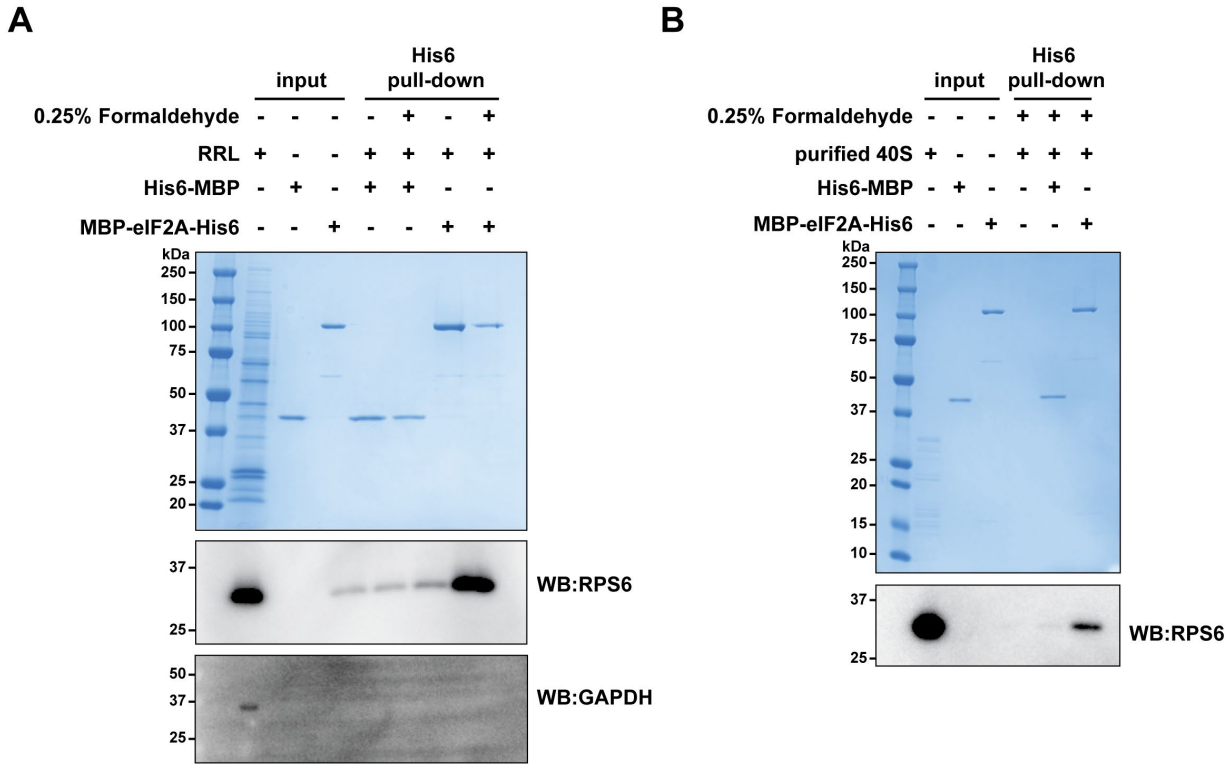


Figure 6. eIF2A directly binds 40S subunits. A) SDS-PAGE and Western blot analysis to assess the ability of recombinant His6-MBP or MBP-eIF2A-His6 (3 µg) to pulldown 40S subunits form RRL with Ni^{2+}-NTA magnetic beads. 0.25% (v/v) formaldehyde was included in the indicated samples to capture transient interactions. RPS6 was used as a marker for 40S subunits. GAPDH was used as a negative control. Coomassie staining was used to confirm capture of His6-tagged recombinant proteins. B) Same as in A, but 0.9 µM purified 40S subunits were used instead of RRL. RPS6 was used a marker for 40S subunits.

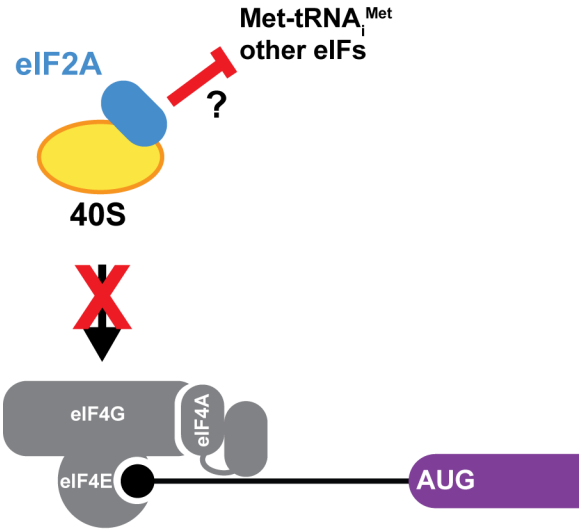
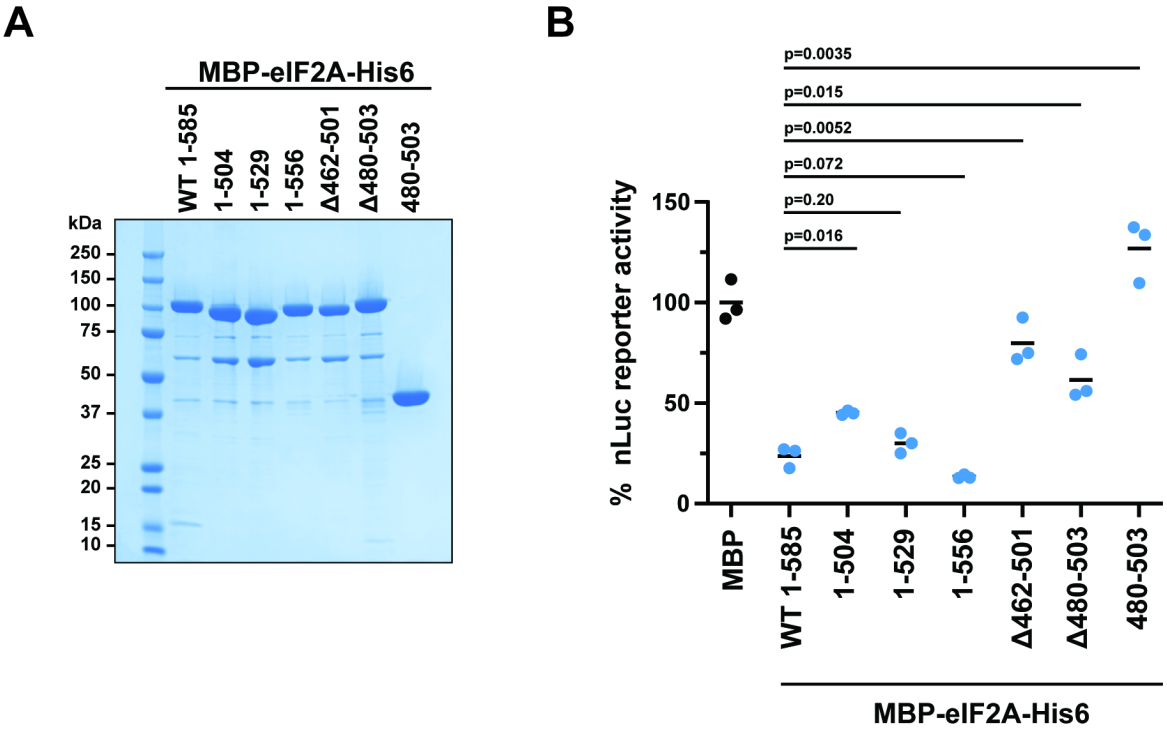
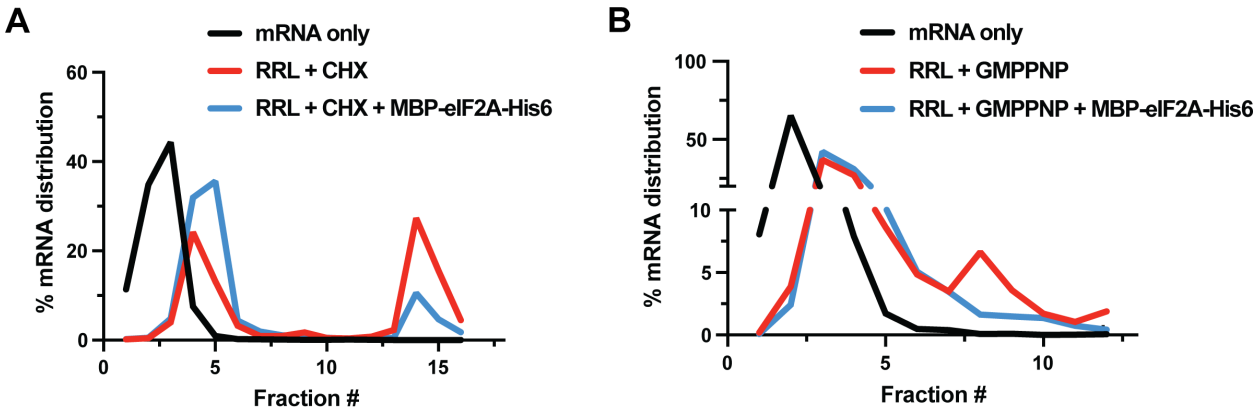


Figure 7. eIF2A inhibits translation by sequestering 40S subunits independent of initiation factors and initiator tRNA. eIF2A directly binds and sequesters the 40S subunit to repress translation. While eIF2A-mediated inhibition only requires the 40S subunit, it remains to be determined if eIF2A blocks or displaces any of the canonical initiation factors or the initiator tRNA.

SUPPLEMENTAL FIGURES



Supplemental Figure S1. Single domain mutants of eIF2A are not translationally repressive. A) SDS-PAGE and Coomassie stain of recombinant MBP-eIF2A-His6 mutants. 2 µg of protein loaded. B) Response of *in vitro* nLuc mRNA translation with the indicated eIF2A mutants (1.68 µM). Bars represent the mean. n=3 biological replicates. Comparisons were made using a two-tailed unpaired t-test with Welch's correction.



Supplemental Figure S2. eIF2A represses translation initiation before 48S initiation complex formation. Replicate gradients as shown in **Figure 4**. A) nLuc mRNA distribution along a 5-30% sucrose gradient supplemented with 100 µg/mL cycloheximide (CHX) to stall 80S ribosomes after one elongation cycle and with either His6-MBP or MBP-eIF2A-His6 (2.0 µM). B) Same as in A, but additionally supplemented with 5 mM GMPPNP to capture 48S initiation complexes at the start codon.

SUPPLEMENTAL INFORMATION

Table S1. Values for heat map in Figure 1.

Cell Line	Tissue/Cell Type	eIF2α (ppm)	eIF2β (ppm)	eIF2γ (ppm)	eIF2A (ppm)
HeLa	Cervical	213	204	110	198
A549	Lung	214	196	109	105
HEK293	Kidney	182	178	92.5	167
MCF7	Breast	206	191	102	190
U2OS	Bone	204	181	96.7	229
Jurkat	T lymphocyte	217	213	109	250
LnCap	Prostate	202	194	102	185
RKO	Colon	233	208	117	210
HepG2	Liver	226	213	114	202

Table S2. Oligonucleotides used in this study.

Oligo Name	Sequence (5'-3')
F_nLuc qPCR	CAGCCGGCTACAACCTGGAC
R_nLuc qPCR	AGCCCATTTTCACCGCTCAG
F_control FFLuc qPCR	AACGCTTCCATCTTCCAGGG
R_control FFLuc qPCR	CCAGATCCACAACCTTCGCT

Reporter mRNA sequences

T7 promoter
start codon
3XFLAG
nLuc
RLuc
stop codon
Hairpin
IRES

nLuc-3XF
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GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATG
ACAAGTAAAAGGGTCAAGACAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTC
TAGAGGGCCC

Recombinant protein sequences
MBP-tag
TEV protease site
His6-tag
eIF2A
FLAG-tag

His6-MBP DNA sequence
ATGGGTTCTTCTCACCATCACCATCACCATGGTTCTTCTATGAAAATCGAAGAAGGTAAACT
GGTAATCTGGATTAACGGCGATAAAGGCTATAACGGTCTCGCTGAAGTCGGTAAGAAATTC
GAGAAAGATACCGGAATTAAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCC
CACAGGTTGCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTTG
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CGCTGTTGAAGCGTTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACC
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GAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACC
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CCTTCAAGGGTCAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCG
CCAGTCCGAACAAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGG
TCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGA
AGAGTTGGCGAAAGATCCACGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAAT

CATGCCGAACATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAA
CGCCGCCAGCGGTCGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTAATGGGA
TCGAGGAAAACCTGTACTTCCAATCCAATATTGGAAGTGGATAA

His6-MBP protein sequence

MGSSHHHHHHGSSMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQ
VAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALS
LIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIK
DVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNY
GVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKS
YEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNGIE
ENLYFQSN

Full length human eIF2A (MBP-eIF2A-His6) DNA sequence

ATGGGTTCTTCTATGAAAATCGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAG
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CGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTTGCGGCAACTGGCGATGG
CCCTGACATTATCTTCTGGGCACACGACCGCTTTGGTGGCTACGCTCAATCTGGCCTGTTG
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TAA

MBP-eIF2A-His6 protein sequence
MGSSMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDI
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mEGFP-FLAG DNA sequence
ATGGGTTCTTCTCACCATCACCATCACCATGGTTCTTCTGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTC
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mEGFP-FLAG protein sequence
MGSSHHHHHHGSSVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEV
KFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQ

LADHYQQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKGS
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MBP-eIF2A-His6-FLAG DNA sequence

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AGCTGGAAGATTTGGAATTGGGTATTGGTTCTTCTCACCATCACCATCACCATGGAGGATC
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MBP-eIF2A-His6-FLAG protein sequence

MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWA
HDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKT
WEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAG
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MENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNGIEENLYFQSNAAPST
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LHSFDLLKAVCLEFSPKNTVLATWQPYTTSKDGTAGIPNLQLYDVKTGTCLKSFIQKKMQNWCP
SWSEDETLCARNVNNEVHFFENNNFNTIANKLHLQKINDFVLSPGPQPYKVAVYVPGSKGAPS
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TNGESAVVQLPKNGPIYDVVWNSSSTEFCAVYGFMPAKATIFNLKCDPVFDFGTGPRNAAYYS
PHGHILVLAGFGNLRGQMEVWDVKNYKLISKPVASDSTYFAWCPDGEHILTATCAPRLRVNNG
YKIWHYTGSILHKYDVPSNAELWQVSWQPFLDGIFPAKTITYQAVPSEVPNEEPKVATAYRPPA
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APQSTPRNTVSQSISGDPEIDKKIKNLKKKLKAIEQLKEQAATGKQLEKNQLEKIQKETALLQELE
DLELGIGSSHHHHHHGGSDYKDDDDK