Rocaglamide A (RocA) typifies a class of protein synthesis inhibitors that selectively kill aneuploid tumour cells and repress translation of specific messenger RNAs1–4. RocA targets eukaryotic initiation factor 4A (eIF4A), an ATP-dependent DEAD-box RNA helicase; its messenger RNA selectivity is proposed to reflect highly structured 5′ untranslated regions that depend strongly on eIF4A-mediated unwinding5. However, rocaglate treatment may not phenocopy the loss of eIF4A activity, as these drugs actually increase the affinity of specific messenger RNAs1–4. RocA targets eukaryotic initiation factor 4E/G/A14,15, represses a subset of these Hipp-sensitive mRNAs (Extended Data Fig. 4a–e). The mTOR inhibitor PP242, which inhibits formation of eIF4F (a complex of eIF4E/G/A)14,15, represses a subset of these Hipp-sensitive mRNAs (Extended Data Fig. 4f, g). Thus, RocA exerts effects beyond reduced eIF4A activity, particularly at low, therapeutic doses.

We next asked how RocA affected eIF4A occupancy across the transcriptome in cells by sequencing transcripts that co-purified with streptavidin binding peptide (SBP)-tagged eIF4A (Extended Data Fig. 5) (RNA-immunoprecipitation sequencing (RIP-seq)). Increasing translation -fold change to RIP -fold change with RocA treatment. a

Indeed, we found that the eIF4A inhibitor hippuristanol (Hipp), which decreases the affinity between eIF4A and RNA12,13, yields a different spectrum of mRNA-specific repression (Extended Data Fig. 4a–e). The mTOR inhibitor PP242, which inhibits formation of eIF4F (a complex of eIF4E/G/A)14,15, represses a subset of these Hipp-sensitive mRNAs (Extended Data Fig. 4f, g). Thus, RocA exerts effects beyond reduced eIF4A activity, particularly at low, therapeutic doses.

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Because RocA enhances the RNA affinity of eIF4A1,2,6, we suspected that it could induce effects beyond the simple loss of eIF4A activity.

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Figure 1 | RNA sequence selectivity is imparted upon eIF4A by RocA causing selective translation repression. a, Histogram of the number of transcripts along translation -fold change by ribosome profiling when cells are treated with 0.03, 0.3, or 3 μM RocA, normalized to the number of mitochondrial footprints. Median -fold change is shown. Bin width is 0.1. b, MA plot of mean footprint reads between 3 μM RocA treatment and non-treatment normalized to library sizes versus translation -fold change by 3 μM RocA treatment, highlighting high- and low-sensitivity mRNAs. c, The 5′ UTRs of indicated genes were fused to Renilla luciferase and these reporter mRNAs were transfected before treatment with RocA as indicated. Data represent mean and s.d. (n = 3). d, Correlation of translation -fold change to RIP -fold change with RocA treatment. ρ, Spearman’s rank correlation.

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Figure 2 | RNA Bind-n-Seq and iCLIP reveal that RocA preferentially increases the affinity of eIF4A and polypurine motif.
a. Correlations between tetramer motif enrichment in Bind-n-Seq by 0.03 μM RocA treatment and motif prediction of 0.03 μM RocA effect in RIP-seq.
b. Highest-scoring elements in Bind-n-Seq and RIP-seq. c. The change in mRNA binding for mRNAs with or without the enriched tetramer motif (b) in their 5′ UTRs is shown as the RIF -fold change by RocA normalized to spike-in RNA. Significance is calculated by Mann–Whitney U-test.
d. Enrichment of tetramer motifs (b) in iCLIP by RocA treatment relative to control dimethylsulfoxide (DMSO) treatment. e. The frequency of the tetramer motif (b) in the 5′ UTR predicts whether a mRNA is high- or low-sensitivity, on the basis of the difference in cumulative distributions of motifs in the 5′ UTR. Significance is calculated by Mann–Whitney U-test.
f. Reporter assay in HEK 293 cells with a CAA-repeat 5′ UTR containing seven polypurine motif (AGAGAG) insertions (Extended Data Fig. 9a). Data represent mean and s.d. (n = 3).

RocA doses elevated the overall amount of RNA that co-purified with SBP-tagged eIF4A (Extended Data Fig. 5d), and greatly changed the abundance of individual transcripts, leading to 15-fold or larger differences between mRNAs. Strikingly, enhanced eIF4A binding in the presence of RocA correlated strongly with translation inhibition by RocA (Fig. 1d and Extended Data Fig. 5f), suggesting that a selective increase of the eIF4A–RNA affinity underlies the specific translation inhibition caused by RocA.

This mRNA selectivity led us to explore the sequence preferences of eIF4A in the absence and presence of RocA. We measured the RNAs that bound to eIF4A out of a random pool of oligonucleotides using deep sequencing (RNA Bind-n-Seq)16 (Extended Data Fig. 6a–c). We then calculated the enrichment of 4- to 6-nucleotide (nt) motifs in mRNAs retained on eIF4A, as DEAD-box RNA helicases typically contact 6 nt (ref. 17). The motifs enriched from randomised synthetic RNA by Bind-n-Seq also predicted RIP-seq enrichments of endogenous transcripts (Fig. 2a and Extended Data Fig. 6d). In both experiments, RocA greatly enhanced binding to short polypurine sequences (Fig. 2b, c and Extended Data Fig. 6e). Although drug-free eIF4A also had intrinsic RNA sequence preferences18 (Extended Data Figs 6g) and transcripts containing these preferred sequences were relatively resistant to H IPP treatment (Extended Data Fig. 6h), RocA only selectively increases binding to a subset of sequences containing polypurine stretches (Extended Data Fig. 6g).

Polypurine motifs were also enriched in the eIF4A binding sites detected by photocrosslinking and immunoprecipitation (iCLIP)18 after RocA treatment (Fig. 2d and Extended Data Fig. 7), and in the 5′ UTRs of translationally RocA-sensitive mRNAs (Fig. 2e). This striking correspondence among in vitro binding to recombinant protein, ex vivo co-purification, crosslinking in cells, and translational repression in cells led us to hypothesise that selective binding to polypurine motifs induced by RocA binding could explain mRNA-specific translational repression. We then directly confirmed that inserting the polypurine motif into an unstructured CAA repeat 5′ UTR (Extended Data Fig. 9a)20 sensitized the reporter to RocA inhibition (Fig. 2f).

Figure 3 | RocA clamps eIF4A on polypurine motif even after ATP hydrolysis. a. b. Direct measurement of the eIF4A/RNA affinity by fluorescence polarization for eIF4A and 5′ FAM-labelled RNAs in the presence or absence of RocA. Data represent mean and s.d. (n = 3). c. Motif enrichments along entire tetramer motifs in Bind-n-Seq with ADP + Pi and highest-scoring elements (inset). d. Competition assay with unlabelled RNA. Data represent mean (n = 3). e. Ribosome toeprinting assay performed in RRL in the presence of GMP-PNP in the presence or absence of 3 μM RocA treatment. f. Relative RNase I cleavage protected by eIF4A/RocA complex on mRNA containing one AGAGAG at the middle in footprinting assay. See the original data in Extended Data Fig. 9g.

We found that RocA-induced, sequence-selective eIF4A binding occurs through ATP-independent clamping that suffices to repress translation of the clamped mRNA. The cycle of ATP-dependent RNA binding and subsequent release upon ATP hydrolysis is necessary for the efficient RNA remodelling activity of eIF4A as well as its role in translation21. Drug-free eIF4A bound RNA only in the presence of ATP (AMP-PNP and ADP-BeF₃) and transition state (ADP-AlF₄) analogues but not hydrolysis products (ADP + Pi). Remarkably, RocA clamped eIF4A on polypurine RNA, but not CAA-repeat RNA, in an ATP-independent manner (Fig. 3a, b and Extended Data Fig. 8a, b). Bind-n-Seq performed with ADP + Pi likewise recovered polypurine-enriched mRNAs in the presence of RocA and no detectable RNA in the absence of RocA (Fig. 3c and Extended Data Fig. 6i). RocA provided polypurine-specific RNA binding activity to mutant eIF4A defective for ATP binding (Vₓ Gly)₂₅, which does not bind to RNA at all without RocA (Extended Data Fig. 8d–f), and even to the truncated amino (N)-terminal domain of eIF4A, albeit with lower affinity (Extended Data Fig. 8g). The eIF4A/RocA complex dissociated far more slowly from polypurine RNA than naive eIF4A, even in the presence of ATP, whose hydrolysis ordinarily permits rapid dissociation (Fig. 3d). High RNA affinity in the ADP-bound state can prolong RNA binding beyond the time required for adenosine nucleotide exchange to restore the high-affinity ATP-bound state and thus greatly reduce the effective dissociation rate. This effective dissociation rate from polypurine RNA measured in hydrolysable ATP (reflective of the intracellular environment) becomes much slower than the ~1 min timescale of translation initiation22, and could serve to directly block the ribosome.

To probe how clamped eIF4A repressed translation, we recapitulated RocA-induced, polypurine motif-specific translational repression
In vitro translation

Supplemental eIF4A

Hipp or 3 μM RocA

Polypurine motifs

Membrane

In vitro translation

Pre-incubation

eIF4A – –

RocA + –

Size-exclusion to remove free RocA

Supplemental eIF4A + + –

In vitro translation

Figure 4 | eIF4A/RocA complexes on polypurine motifs block scanning of pre-initiation complex, inducing uORF translation. a, Pre-formation of the complex with RocA and eIF4A on the mRNA bearing seven polypurine motifs represses the translation from the mRNA in RRL. b, The supplementation of recombinant eIF4A protein to RRL in vitro translation reaction with 10 μM Hipp or 3 μM RocA. c, In vitro translation in RRL with mRNAs with native polio virus IRES and that with three polypurine motifs (Extended Data Fig. 9a). d, Meta-gene analysis of high-sensitivity transcripts to RocA. Reads are normalized to the sum of mitochondrial footprints reads. Histogram of the position of the first polypurine motif (hexamer) after uORF initiation codon (inset). P value is calculated by Fisher’s exact test. Bin width is 12 nt. e, Western blot of SBP translated from uORF and downstream major ORF in RRL with 0.03 μM RocA treatment. Quantification of bands normalized to long form with DMSO treatment is shown. For gel source data, see Supplementary Fig. 1. f, Schematic representation of RocA-mediated translation control. RocA clamps eIF4A onto mRNA by selective affinity enhancement for a polypurine motif in eIF4F-, cap-, and ATP-independent manners, which then blocks scanning of pre-initiation complex, introducing premature translation from uORF and inhibiting downstream ORF translation. In b and c, data represent mean and s.d. (n = 3).

translated from uORF and downstream major ORF in RRL with 0.03 μM RocA treatment. Quantification of bands normalized to long form with DMSO treatment is shown. For gel source data, see Supplementary Fig. 1. f, Schematic representation of RocA-mediated translation control. RocA clamps eIF4A onto mRNA by selective affinity enhancement for a polypurine motif in eIF4F-, cap-, and ATP-independent manners, which then blocks scanning of pre-initiation complex, introducing premature translation from uORF and inhibiting downstream ORF translation. In b and c, data represent mean and s.d. (n = 3).
can drive cryptic upstream initiation using a reporter mRNA with two alternative start sites that yield distinguishable protein isoforms. Insertion of a polypurine motif 30 nt downstream of the earlier AUG increased translation initiation from this codon upon RocA treatment (Fig. 4e), confirming that clamped eIF4A/RocA complexes on polypurine motifs drive upstream translation initiation. We found evidence that this enhanced upstream initiation could contribute to eIF4A/RocA-mediated repression of downstream CDSes58, as RocA-sensitive transcripts showed more pre-existing uORF initiation29 (Extended Data Fig. 10d, e).

We have shown that RocA induces ATP-independent clamping of eIF4A onto polypurine sequences, creating an inhibitory roadblock for the scanning ribosome (Fig. 4f). Our identification of the eIF4A/RocA binding motif provides the first observation of a drug that stabilizes sequence-selective RNA–protein interactions60. RocA may bind near the RNA interface on the N-terminal domain of eIF4A6, raising the possibility that the drug directly contacts purine bases of target RNAs. Alternatively, RocA might induce a conformational change leading to direct or indirect recognition of the polypurine motif by protein residues. Future structural insight into this polypurine selectivity may enable rocalgale derivatives with altered base selectivities that target different mRNA.
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

General methods. HEK 293 Flp-In T-Rex cells (Invitrogen) were cultured and recombined according to manufacturer’s instructions. Stable integrants of SBP-tagged eIF4A were produced by co-transfection of these plasmids along with pOG44 by X-tremeGENE 9 (Roche) and selection using Hygromycin B. RocA, PP242, and Qiazo1 (Qiagen) were purchased from Sigma. Proteins or RNA/DNA/RNA were stained with GelCode Blue Stain Reagent (Thermo Scientific) and SYBR Gold Nucleic Acid Gel Stain (Invitrogen), respectively.

Ribosome profiling. Library preparation and data analysis were performed according to the method previously described31, which monitors mitochondrial ribosomes as well31, DMSO, RocA, Hpp, and PP242 were added to medium 30 min before cell lysis. The libraries were sequenced on a HiSeq2000/2500 (Illumina).

RIP-seq. Cells with cytotoxic-inducible, SBP-tagged eIF4A integrated stably were plated in a 10 cm dish and cultured for 3 days with 1 μg/ml tetraclit, incubated with DMSO. 0.03 μM or 0.3 μM RocA for 30 min, washed once with 5 ml of ice-cold PBS, lysed with 600 μl of lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM MgCl2, and 1 mM EDTA) containing 1% Triton X-100 and Turbo Dnase I (Invitrogen) 25 U ml−1, and then clarified by centrifugation at 10 min at 20,000g. 4 °C. The supernatant was incubated with 60 μl of Dynabeads M-270 Streptavidin (Invitrogen) equilibrated with lysis buffer containing 1% Triton X-100 at 4 °C for 30 min. The beads were washed twice with lysis buffer containing 1% Triton X-100 and 1 M NaCl. SBP-eIF4A and bound RNAs were eluted with 25 μl of lysis buffer containing 5 mM NaBi at 4 °C for 30 min. All buffers contained 0.01% DMSO with or without 0.03 or 0.3 μM RocA. RNAs were extracted with Qiazo1 (Qiagen) using the Direct-zol RNA miniprep (Zymo Research). One-third of eluted RNA (~100 ng) was mixed with 1 ng of in vitro transcribed, spike-in Renilla luciferase RNA (rHLuc) (see ‘DNA constructs’) and sequencing libraries were prepared using Tru-seq Ribo-zero gold kit (Illumina). Libraries were sequenced on HiSeq2000/2500 (Illumina) sequencers.

iCLIP. Cells were cultured as described in ‘RIP-seq’. After medium was substituted with ice-cold PBS, the dishes on ice were irradiated with 150 μm C−UV-C (~254 nm) in UVP CL-1000 (UVP), Lyaste was prepared as described in ‘RIP-seq’. The lyaste from a 10 cm dish (600 μl) was treated with 0.4 μg of RNase I (Epicentre) at 37 °C for 3 min. Reaction was quenched by the addition of 10 μl of SuperRa RNA Inhibitor (Invitrogen), and RNase was treated with 0.4 U of RNase I (Epicentre) (see ‘DNA constructs’) and sequencing libraries were prepared using Tru-seq Ribo-zero gold kit (Illumina). Libraries were sequenced on HiSeq2000/2500 (Illumina) sequencers.

Data analysis. The reads were aligned to the hg19 human genome reference and the resulting aligned reads were mapped to University of California, Santa Cruz (UCSC) known reference genes, downloaded from the UCSC genome browser in July 2013. A UCSC bed file of known genes was used for the 5′ UTR analysis. For mitochondria footprints alignments, we used the RefSeq genes track corresponding to the mitochondrial chromosome (chrM), downloaded from UCSC genome browser. Specific A-site nucleotides were empirically estimated on the basis of the length of each footprint. The offsets were 14 for 26–29 nt and 15 for 30–31 nt. For mitochondria footprints, they were 9 for 26–27 nt, 11 for 28–29 nt, 12 for 30 nt, 13 for 31 nt, and 18 for 32–34 nt. For mRNA fragments, we used offset 14. For measuring footprint density and mRNA fragments of RIP-seq between samples, we restricted our analysis to genes, which have at least 40 and 100 summed counts in each sample, respectively. For CDSs, the analysis only included the transcript positions beginning 15 codons following the start codon and stopping 5 codons preceding the stop codon. For 5′ UTRs, we included the transcript positions from the 5′ end of the mRNA until 5 codons preceding the start codon. DESeq2 was used to calculate relative enrichment of genes in the library, including the mitochondrial footprints and spike-in rHLuc mRNA counts. The calculated -fold change was re-normalized to the value of the summed mitochondria footprints or the spike-in rHLuc mRNA fragments. The low-sensitivity transcripts were defined as transcripts with reduction more than twofold from the median, and with q value < 0.01, between 3 μM RocA-treated and untreated cells. Low-sensitivity transcripts are defined as same as high-sensitivity but with accumulation over twofold.

For calculation of ΔG, RNAfold (ViennaRNA Package)33 was run with –L30 –g –Q 10 options on 5′ UTR sequences from UCSC foldUtr5 table. The minimum ΔG along each 5′ UTR was used as a representative free energy value for each gene.

The presence of G-quadruplexes was predicted with RNAfold (ViennaRNA Package).

The Gini differences across 5′ UTRs were calculated using published data11. Analysis was restricted to the mRNAs bearing 5′ UTRs having one or more reads on A/C positions on average.

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above, as the great majority of these footprints derive from ribosomes trapped on first codons (Extended Data Fig. 10d). To normalize mRNA abundance in cells, we normalized the density by footprint counts from the CDS start codon region using the genomic position between start codon and 6 nt downstream. We restricted the analysis to transcripts with at least 10 counts from CDS start codons and re-normalized the value to the median as one.

Code availability. Scripts to run the analyses mentioned above are available upon request.

Fluorescence polarization assay. Proteins (0–50 μM) were incubated in 14.4 mM HEPES-NaOH, 108 mM NaCl, 1 mM MgCl₂, 0.36 mM TCEP, 14.4% glycerol, 0.1% DMSO, and 10 mM 5’-FAM-labelled RNA with or without 50 μM RocA in 10 μl reaction for 30 min at 25 °C. The experiments were performed with 1 μM AMP-PNP (for AMP-PNP, 1 μM ADP, 5 mM BeCl₂, and 25 mM NaF for (ADP-BeF), 1 mM ADP, 5 mM AlCl₃, and 25 mM NaF (for ADP-AlF₃), or 1 mM ADP and 1 mM Na₂HPO₄ (for ADP + Pi). For the condition without ATP analogue, MgCl₂ was omitted from the reaction.

For competition assay, the complexes were preformed with 1 μM ATP or AMP-PNP, 1 μM eIF4A, 10 μM FM-labelled RNA, and 50 μM RocA and chased with 100 μM non-labelled RNA. Because of the low affinity, 50 μM eIF4A was used with ATP and DMSO.

Fluorescence polarization was measured using an Infinite F-200 PRO (TECAN). The dissociation constant (K_d) and half-life (t½) were calculated with fitting to the Hill equation and one-phase exponential decay equation, respectively, by Igor Pro software (WaveMatrics).

In vitro translation and toeprinting assay. In vitro translation was performed with nucleic-acid-treated RRL system (Promega), according to the manufacturer’s instructions. Reporter mRNAs (50 nM; see ‘DNA constructs’) was incubated in 50% RRL with RocA (concentration shown in the figure legends) or 1% DMSO in 10 μl at 30 °C. For the detection of S6 293 μl of the reaction was used with uORF + CAACAA or uORF + AGAGAC mRNAs and concentrated with 10 μl of Dynabeads M-270 Streptavidin (Invitrogen).

The toeprinting assay was performed as previously described. The reaction was pre-incubated with RRL in the presence of 2 mM GMP-PNP or m7GTP and 3 μl RoA or 1% DMSO at 30 °C for 5 min, and then incubated with 30 nM mRNAs at 30°C for 5 min, followed by reverse transcription with 10 U M-MLV Reverse Transcriptase II (NEB) with 250 nM 5’-6-FAM labelled primer (5’-6-FAM-ATGCAGAAAAATCACGGC-3’) at 30 °C for 15 min. Recombinant eIF4A (10 μM) was used instead of RRL in 30 μl mHEPES, pH 7.3, 100 mM KOAc, 1 mM Mg(OAc)₂, and 1 mM DTT in the presence or absence of 10 μM RocA. Complementary DNA (cDNA) was purified by phenol extraction, concentrated using Oligo Clean & Concentrator (Zymo Research), and run with GeneScan 600 LIZ Size Standard v2.0 (Life Technologies) on a 3730 DNA Analyzer (Life Technologies). Data were analysed by GeneMapper software (Life Technologies). For preformation of eIF4A/RocA complex on mRNA, 30 μl of the reaction was used on 3730 DNA Analyzer as transcription template.

Dideoxy-terminating sequencing of RNA was performed by reverse transcriptions using 0.125 mM individual dideoxy-NTP and 0.5 mM each deoxy-NTP with the same 5’-6-FAM-labelled primer and Protocol II, according to the manufacturer’s instructions.

RNAse I footprinting assay. Reporter RNA was incubated with recombinant eIF4A and RocA in 12 μl as described for the toeprinting assay. The reaction was treated with 1 μl of 0.001 U μl⁻¹ RNAse I (Epicenter) at room temperature for 5 min. After quenching the digestion by the addition of 1 μl of SUPERase In RNAse Inhibitor (Invitrogen), RNA was extracted using Oligo Clean & Concentrator (Zymo Research) and reverse transcribed by Protocol II (NEB) with 5’-6-FAM labelled primer (5’-6-FAM-ATGCAGAAAAATCACGGC-3’) according to the manufacturer’s instructions. The cDNA was run on 3730 DNA Analyzer (Life Technologies) as described for the toeprinting assay. Data were analysed by GeneMapper software (Life Technologies).

Polysome profiling. Cell lysate was prepared as described previously. Lysate containing 15 μg total RNA was loaded on to 10–50% linear sucrose gradients containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 100 μg ml⁻¹ cycloheximide, and 2 μl 1 μl⁻¹ SUPERase In RNAse Inhibitor and sedimented by ultracentrifugation at 220,000g for 2 h at 4°C with an SW41 rotor (Beckman Coulter). Gradients were fractionated using Gradient station (Biocomp). Ultraviolet absorbance was detected by ECONO UV monitor (Biorad).

Metabolic labelling of nascent peptide by OP-puro. Nascent peptides in HEK 293 cells were labelled by 40 μM OP-puro (Jena Bioscience) in 24-well dishes with 0–3 μl RocA for 30 min. Cells were washed with PBS and lysed with 50 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, and 1 mM DTT) containing 1% Triton X-100, and then clarified by centrifugation with 20,000g at 4°C for 10 min. Nascent peptides were labelled with 5 μl Alexa Fluor 488 Azide (ThermoFisher Scientific) by a Click-it Cell Reaction Buffer Kit (ThermoFisher Scientific) according to the manufacturer’s instructions and run on SDS–PAGE. Images were acquired by FluorChem R imaging system (ProteinSimple) and quantified by AlphaView (ProteinSimple).

DNA constructs. DNA fragments containing 5’ UTRs sequences, listed below, were inserted between T7 promoter and ORF of Renilla luciferase (hRluc) in psiCHOP (Promega). We cloned exactly the same sequence of G-quadruplex and its control sequence used in ref. 5. These plasmids were digested by NotI and used as in vitro transcription template.
Polio virus IRES 3× was AGAGAG: TTAAAACAGCTCTGGGGTTGTA

TTATTTTATGTGGCTCTATGTCAGCACAACATCAAAAAGGAGGAAGGAAGGAGG

TTGATGACTCGAGACCTCTCAAACGTAAACCAAACAACAAGCAGTGACAC

TTTGATGACTCGAGACCTCTCAAACGTAAACCAAACAACAAGCAGTGACAC

Reverse transcriptions were performed with ProtoScript II (NEB) and random primers (NEB) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with Fast EvaGreen qPCR Mix (Biotium) in BioRad CFX96 Touch Real Time PCR Detection System (Bio-Rad) with oligonucleotides, 5′-TGTCCTGATCTGAGAGTGTGTC-3′, and 5′-CTAACCTGCCCTTCTCCTT-3′. RNA from non-transfected cells or in vitro translation reaction without addition of mRNAs was used as qPCR background.

**Purification of recombinant eIF4A proteins.** Typically, BL21 Star (DE3) Escherichia coli cells (Invitrogen) were transformed with pHisMBP-eIF4A, pHisMBP-eIF4A (VX 4 GKT) (D296A-T298K), or pHisMBP-eIF4A (1-237) in 1.5 L culture were cultivated to an absorbance of 0.4 nm, A600nm, of 0.3 at 37°C with 50 μg ml⁻¹ kanamycin and then grown at 16°C overnight with 1 mM IPTG. The cell pellets were resuspended in His buffer (20 mM HEPES-NaOH, pH 7.5, 50 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol) with 0.5% NP-40, sonicated, and centrifuged at 35,000g for 30 min. The supernatant was incubated with 1 ml bed volume of Ni-NiTA Superflow (Qiagen) for 1 h. The beads were loaded on a gravity column and washed with His buffer containing 1 M NaCl. The proteins were eluted with 50 mM Na-phosphate buffer, pH 7.5, 500 mM NaCl, 10 mM Na2SO4, 230 mM imidazole, 10 mM β-mercaptoethanol, treated with tobacco etch virus protease overnight, dialysed to 20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 0.5 mM TCEP, and 10% glycerol, and loaded on MBrP Trap HP 5 ml (GE Healthcare). The flow-through fractions were collected, concentrated with Amicon Ultra 10kDa (Millipore), and loaded onto a HiLoad 16/60 Superdex 75 prep grade column (GE Healthcare) equilibrated with 20 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.5 mM TCEP. The peak fractions were collected, concentrated with Amicon Ultra 10kDa (Millipore), mixed with 0.25 volumes of 80% glycerol, shock-frozen in liquid nitrogen, and stored at −80°C. All purification steps were performed at 4°C. Chromatography was performed using an AKTA purifier (GE Healthcare).

**Pulldown assay.** The lysate of E. coli cells expressing eIF4A wild type (WT) or eIF4A D296A-T298K proteins was used in 'Purification of recombinant eIF4A proteins' and incubated with 10 μl of HisPur Ni-NiTA Magnetic Beads (Thermo Scientific) at 4°C for 30 min. The beads were washed five times with His buffer containing 1 M NaCl, rinsed once with 20 mM HEPES-NaOH, pH 7.5, 10 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, and incubated with RRL (Promega) at 25°C for 30 min. After five washes with His buffer, the proteins were eluted from the beads by SDS sample buffer.

**ATP crosslinking assay.** Recombinant eIF4A WT and VX 4 GKT (10 μM) was incubated with 1 μM [γ-32P]-ATP (3,000 Ci mmol⁻¹, Perkin Elmer) in 30 mM Hepes-KOH, pH 7.3 (Fisher Scientific), 100 mM KOAc, 5 mM Mg(OAc)2, and 1 mM DTT in 20 μl reaction at 37°C for 30 min. The reactions were exposed to 1500 mJ cm⁻² using UV254 nm (CL-1000, UV) at a distance of 2 cm from the lamp on ice and run on SDS—PAGE. The images were acquired by Typhoon TRIO (Amersham Biosciences).

**Western blotting.** Anti-eIF4A1 (2490, Cell Signaling) (1:1,000), anti-phospho-eIF2α (Ser51) (D9G8 3398, Cell Signaling) (1:1,000), anti-4EBP (1-237), or pHisMBP-eIF4A (VX 4 GKT) (A82V) and D296A-T298K mutations were introduced by site-directed mutagenesis.

**Reportase assay in HEK 293 cells.** Transfections were performed in 24-well dishes with a TransIT-mRNA Transfection Kit (Mirus) according to the manufacturer's instructions, at half scale. Three hours after transfection, RocA was added to the in vitro translation reaction for luciferase assay.

**Quantitative PCR.** Primer was designed to construct pHisMBP-eIF4A, VX 4 GKT (A82V) and D296A-T298K with the help of ViennaRNA Package 2.0. The complementary DNA was generated by reverse transcription of ribosome-protected mRNA fragments.

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Extended Data Figure 1 | RocA represses translation, targeting to eIF4A. a, Polysome profiling experiments with RocA and PP242 treatments. RocA disrupts polysomes dose-dependently. b, Western blot of phospho-eIF2α and phospho-4E-BP shows that effect of RocA is independent of known translation control targeting to eIFs. Phosphorylation of eIF2α and dephosphorylation of 4EBP were induced by thapsigargin and PP242, respectively. c–d, Luciferase reporter assay possessing PTGES3 5′ UTR (Fig. 1c) with exogenous expression of WT or RocA-resistant eIF4A mutants (c) and western blot of endogenous and exogenous eIF4A (d). eIF4A is the main molecular target of RocA. Data represent mean and s.d. (n = 3). e, f, Correlation of sum of the footprint reads to 13 mitochondrial mRNAs among different conditions (e) and correlation of sum of the footprint reads from cytoplasmic ribosomes to each transcript between biological replicates (f). Symbol r is Pearson’s correlation. P value is calculated by Student’s t-test. g, h, Tile plot of codon periodicity along length of mitochondria footprints (g, left) and mitochondria footprint length distribution (g, right) and codon periodicities of 31-nt mitochondrial footprints among different conditions (h). Footprints with 31-nt length showed most homogenous codon periodicity, and this periodicity was retained with RocA treatment, showing that mitochondrial ribosome translates even in high doses of RocA.
Extended Data Figure 2 | RocA represses translation without mRNA degradation. a, Metabolic labelling of nascent peptides with OP-puro. The OP-puro incorporated nascent peptides were visualized by Click reaction with Alexa Fluor 488 Azide (middle) and quantified (right). Data represent mean and s.d. (n = 3). b, Correlation of translation -fold change among different concentrations of RocA treatments. c, MA plot of mean footprint reads between 0.03 μM RocA treatment and non-treatment normalized to library sizes to footprints -fold change by 0.03 μM RocA treatment (left) and the correlation of translation -fold change between 0.03 and 3 μM of RocA treatments (right), highlighting high-sensitivity mRNAs at 0.03 μM RocA treatment. d, Scatter plots of footprint -fold change normalized to mitochondrial footprints and mRNA -fold change by RocA treatments. RocA represses translation without significant mRNA change. e, qPCR from the samples of Fig. 1c. Data represent mean and s.d. (n = 3).
Extended Data Figure 3  | Secondary structure in 5′ UTR is not strong determinant of RocA sensitivity. a, Cumulative fractions along length of 5′ UTR, minimum ΔG among all 30-mer windows along a 5′ UTR, ΔG in cap-proximal region (30 nt) of 5′ UTR, and Gini difference are plotted to total, RocA high-sensitivity, and RocA low-sensitivity mRNAs. Significance is calculated by Mann–Whitney U-test. b, Cumulative fractions along translation -fold change by RocA are plotted to total mRNAs and mRNAs with predicted G-quadruplexes in 5′ UTRs. Significance is calculated by Mann–Whitney U-test. The impact of presence of G-quadruplex in 5′ UTR is modest in RocA sensitivity. c, The 5′ UTRs with G-quadruplexes and randomized control sequence were fused to Renilla luciferase and these reporter mRNAs were transfected before treatment with RocA as indicated. Data represent mean and s.d. (n = 3). G-quadruplex does not show the prominent RocA sensitivity.
Extended Data Figure 4 | Characterization of translational inhibition by Hippuristanol and PP242. a, Polysome profiling experiments with Hipp treatments. Hipp disrupts polysomes dose-dependently. b, Histograms of number of transcripts along footprints -fold change with 0.01 and 1 μM Hipp treatment compared with non-treatment, normalized to mitochondrial footprints. Median -fold change is shown. Bin width is 0.1. c, MA plot of mean footprint reads between 1 μM Hipp treatment and non-treatment normalized to library size to translation -fold change by 1 μM Hipp treatment, highlighting high-sensitivity and low-sensitivity mRNAs. d, Cumulative fractions along length of 5′ UTR, minimum ΔG among all 30-mer windows along a 5′ UTR, ΔG in cap-proximal region (30 nt) of 5′ UTR, and Gini difference are plotted to total, Hipp high-sensitivity, and Hipp low-sensitivity mRNAs. Significance is calculated by Mann–Whitney U-test. e, Translation -fold changes by RocA and Hipp are modestly correlated. f, MA plot of mean footprint reads between 2.5 μM PP242 treatment and non-treatment normalized to library sizes to translation -fold change by PP242 treatment, highlighting PP242 target mRNAs. g, Cumulative distributions of translation -fold change caused by RocA and Hipp treatment are plotted for total and PP242-target mRNAs. Significance is calculated by Mann–Whitney U-test. © 2016 Macmillan Publishers Limited. All rights reserved.
Extended Data Figure 5 | Purification of SBP-tagged eIF4A and co-purified RNA from HEK 293 cells. a, Western blot of exogenous SBP-eIF4A and endogenous eIF4A in tetracycline-inducible stable cell line. Expression of physiological levels of the tagged allele attenuated endogenous eIF4A expression but preserved overall eIF4A levels, probably reflecting the same feedback loop previously reported between eIF4AI and eIF4AII37. b, CBB staining of purified SBP-eIF4A and SYBR Gold staining of purified RNA bound to SBP-eIF4A with or without micrococcal nuclease (MNase). c, Correlation of sum of the mRNA fragment reads of each transcript between biological replicates of RIP-seq. P value is calculated by Student's t-test. d, Histogram of the number of transcripts along RNA/eIF4A interaction -fold change by RIP-seq when cells are treated with 0.03 or 0.3 μM RocA normalized to spiked-in RNA. Data present the same mRNAs analysed in Fig. 1a. Median -fold change is shown. Bin width is 0.1. e, Correlation of RIP -fold change between different concentration of RocA treatments. f, Correlation of translation -fold change to RIP -fold change with the same concentration of RocA treatment.
Extended Data Figure 6 | Motif enrichment by Bind-n-Seq.

a, Nucleotide composition in each length of reads in input RNAs for Bind-n-Seq. Input RNAs are random in entire read length. b, Length distribution of reads from Bind-n-Seq. RNAs bound to eIF4A showed longer length distribution, indicating that eIF4A has preference for longer RNAs. c, Correlations of tetramer motif enrichment in Bind-n-Seq by 0.03 μM RocA treatment to that by 0.3 μM RocA treatment. d, Correlations between pentamer and hexamer motif enrichment in Bind-n-Seq by 0.03 μM RocA treatment and motif prediction of 0.03 μM RocA effect in RIP-seq. e, Highest-scoring pentamer and hexamer motifs in Bind-n-Seq and RIP-seq. f, Cumulative fractions along number of tetramer motifs (Fig. 2b) in 5′ UTR are plotted to total, RocA high-sensitivity, and RocA low-sensitivity mRNAs. Significance is calculated by Mann–Whitney U-test. g, Correlations of Bind-n-Seq motif enrichment (pentamer) by elf4A to that by 0.03 μM RocA treatment. The motifs appearing in RNAs used in Extended Data Fig. 8 are highlighted. h, Correlation of Bind-n-Seq motif enrichment (pentamer) by elf4A to motif prediction of Hipp effect in translation change, which is defined as Spearman's correlation of motif number in 5′ UTR to translation -fold change by Hipp. mRNAs with high-affinity motif to elf4A in 5′ UTR are resistant to Hipp treatment. i, The correlation between enriched motifs of replicates in Bind-n-Seq with ADP + Pi.
Extended Data Figure 7 | Characterization of iCLIP data.

a, CBB staining of purified SBP-eIF4A protein in iCLIP procedure. 
b, Visualization of RNA-crosslinked with SBP-eIF4A and unknown proteins by 32P labelling of RNA. We avoided the contamination of RNAs cross-linked to the additional, co-purifying, unknown proteins. 
c, Distribution of read length in iCLIP libraries. Avoidance of contaminating RNAs restricted us to short RNAs, which probably correspond to the region of RNA physically protected by eIF4A binding, or footprint. 
d, Nucleotide bias along the reads in iCLIP libraries. The crosslinking bias for U may underestimate the preference for polypurine motifs. 
e, Correlations of iCLIP motif enrichment (tetramer) by different RocA concentrations. 
f, Correlations of iCLIP motif enrichment (tetramer) by 3 µM RocA and motif prediction of 0.03 µM RocA effect in RIP-seq. The motifs shown in Fig. 2a are highlighted.
Extended Data Figure 8 | eIF4A/RNA affinity measured by fluorescence polarization. a, CBB staining of recombinant proteins used in this study. b, Summary of $K_d$ between RNA and eIF4A among the conditions assayed. c–g, i, Direct measurement of the eIF4A/RNA affinity by fluorescence polarization for eIF4A WT, eIF4A (VX4GKT), or eIF4A (D296A–T298K) and 5′ FAM-labelled RNAs in the presence or absence of RocA. Data represent mean and s.d. (n = 3). d, ATP crosslinking assay with eIF4A WT and eIF4A (VX4GKT). h, Pulldown assay with His-MBP-eIF4A expressed in E. coli and eIF4E/G in RRL.
Extended Data Figure 9 | Characterization of toeprinting assay. a, Diagram of the reporters used in this study. b, c, In vitro translation in RRL with mRNAs containing seven polyuridine motif (AGAGAG) insertions (b) and qPCR from the samples (c). d, Dideoxy-terminated sequencing of RNA by reverse transcription verified the toeprinting product length terminated by 48S ribosomes. e, Ribosome toeprinting assay performed in RRL in the presence of m7-GTP in the presence or absence of 3 μM RocA treatment. f, Toeprinting assay using 10 μM recombinant eIF4A in the presence or absence of 10 μM RocA treatment. g, Toeprinting assay (top) and RNase I footprinting assay (bottom) using 10 μM recombinant eIF4A with mRNA containing one AGAGAG motif at the middle in the presence or absence of 10 μM RocA treatment. h, i, Toeprinting assay using 10 μM recombinant eIF4A (VX4GKT) or (D296A-T298K) with mRNA containing seven AGAGAG motifs in the presence or absence of 10 μM RocA treatment. j, Pre-formation of the complex with RocA and eIF4A (VX4GKT) or (D296A-T298K) on the mRNA bearing seven polyuridine motifs represses the translation from the mRNA in RRL. k, Basal translation level from mRNA containing seven AGAGAG motifs with the supplementation of recombinant eIF4A. l, Basal translation level from mRNA bearing poliovirus IRES and poliovirus IRES with three AGAGAG motifs. In b, c, and h−j, data represent mean and s.d. (n = 3).
Extended Data Figure 10 | The 5′ UTR footprints accumulated in RocA treatments come from uORFs. a, The distributions of specific footprint length, which is a hallmark of 80S ribosomes, from CDS and 5′ UTR are indistinguishable. b, The change in ribosome footprint counts for 5′ UTRs and CDSs when cells are treated with 3 μM RocA or 1 μM Hipp compared with non-treatment, normalized to mitochondrial footprints. Median -fold change is shown. Bin width is 0.1. Analysis is restricted to mRNAs bearing footprints in the 5′ UTR in the non-treatment condition. c, Meta-gene analysis of low-sensitivity transcripts to RocA. Reads are normalized to the sum of mitochondrial footprint reads. d, The illustration of the definition of uORF translation intensity. e, Transcripts sensitive to RocA contain more active uORFs, as measured by cumulative distributions of the uORF translation intensity. Significance is calculated by Mann–Whitney U-test. f, The summary of deep sequencing-based approaches used in this study and corresponding figures.