Analysis of translation initiation using a translation control reporter system

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Published online 9 November 2006; doi:10.1038/nprot.2006.274

The study of translational control has become increasingly important, as aberrant translation has been linked to the etiology of human diseases. Nevertheless, a convenient research tool to measure and quantify cellular translational activity has not been developed to date. Here we present a translation control reporter system (TCRS) for straightforward and accurate analysis of cellular translational activity. Our method relies on the expression of two unique reporter peptides from a single messenger RNA transcript. Using TCRS-expressing cell lines, changes in initiation of translation have been detected in response to translationally active drugs. Accordingly, TCRS may promote the discovery of novel agents that modulate translation. TCRS may also be used in the identification of signal transduction pathways that impinge on translation control. Furthermore, the modular design allows the exchange of regulatory cassettes for the examination of other putative *cis*-regulatory mRNA elements. The time required for the procedure depends on whether transient TCRS expression is used or stable TCRS-expressing cell lines have to be produced and will range from 5 to 14 d, respectively.

INTRODUCTION

Regulated translation of mRNA has a major role in animal development and physiology and is involved in the etiology of a number of human diseases[1,2]. Global changes in protein synthesis can occur in response to signaling cascades that sense various signals, including fluctuations in nutrients, growth factors, mitogens and inducers of differentiation. However, the translation of subgroups of mRNAs may be differentially regulated, which is usually achieved by use of *cis*-regulatory sequences in the mRNAs.

Changes in global translational efficiency can be evaluated by comparing total amounts of separated proteins in an SDS-polyacrylamide gel after metabolic radioactive labeling[3]. For more specific analysis of translation, the 5′ untranslated region (UTR) and/or 3′ UTR sequences of the studied mRNA can be used to drive translation of an easily measurable reporter gene (luciferase). In addition, bicistronic reporter constructs that contain firefly luciferase and *Renilla* luciferase cistrons in tandem are used to identify translation from internal ribosomal entry sites (IRES) located in the intercistronic sequences[4]. These conventional reporter constructs allow evaluation of cap-dependent translation levels and, in the case of the bicistronic constructs, comparison with eventual IRES-driven translation. However, with these procedures it is difficult to discriminate between translational effects and changes in transcription and/or mRNA stability. Moreover, such systems cannot evaluate changes in translation reinitiation, which has been shown to be particular sensitive to cellular translational conditions[5–7]. Additional difficulties in interpretation of the signals may arise from unforeseen splicing, cryptic promoters or the kinetics of the amplified luminescence signal[8].

In case of TCRS, the output signal consists of two peptides that are expressed from a single transcript. Therefore, changes in transcription levels and/or mRNA turnover will evenly affect expression levels of both TCRS peptides. Principally, changes in the ratio of expression of the two TCRS peptides reflect differences between translation initiation and reinitiation. The TCRS comes with a negative control construct lacking a functional open reading frame to ensure that translational mechanisms, and not other unrelated gene regulatory mechanisms, are responsible for expression of TCRS peptides. In addition, the obtained results are directly based on actual peptide expression levels and not on amplified luminescence signals.

One class of translation regulatory sequences is small upstream open reading frames (uORFs), which are located in the 5′ UTR of certain mRNAs[9]. The TCRS used in this protocol contains the uORF from the *CEBPA* gene, encoding transcription factor C/EBPα, to control translation from a reporter transcript. In its natural setting, this uORF controls translation from a second downstream AUG codon in the *CEBPA* transcript, which results in the expression of an extra, N-terminally truncated C/EBPα isoform in addition to the full-length C/EBPα isoform that is expressed from the first AUG codon[5]. The translational mechanism involved has been deduced from studies on *CEBPA* but also other genes, especially *GCN4* (ref. 6), and from studies on uORF-mediated translational effects in general[9,10]. In brief, as the *CEBPA* uORF initiation AUG codon is in a suboptimal context, some translating ribosomes recognize and translate the uORF, whereas others do not. Those that have translated the uORF resume 5′→3′ scanning along the mRNA and may participate in translation reinitiation at the downstream site. The efficiency of translation reinitiation is negatively regulated by phosphorylation of the initiation factor eIF2α, which restrains reloading of the ribosome with the ternary eIF2–GTP–Met-tRNA$_i^{Met}$ complex required for initiation of protein synthesis. In addition, the efficiency of reinitiation is sensitive to inhibition of the mammalian target of rapamycin (mTOR) by rapamycin and eIF4E levels. This suggests that, similar to its critical role in preinitiation-complex assembly[11], mTOR is crucial for the stability of the scanning preinitiation complex.

The TCRS comprises three parts: the central expression cassette, the 5′ translation regulation cassette and the 3′ regulatory cassette (see **Fig. 1**). The central peptide expression cassette codes for two

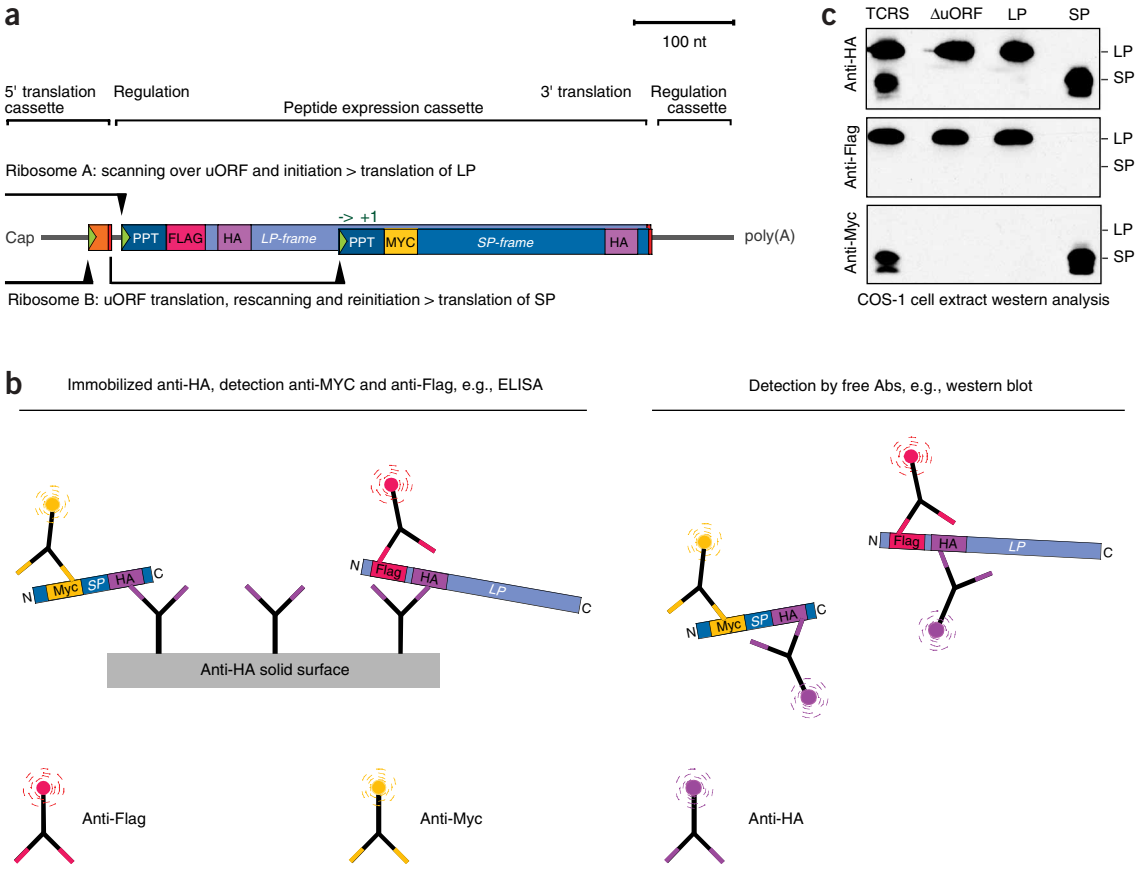

Figure 1 | Design and function of the TCRS. (**a**) The suboptimal context of the uORF initiation codon allows some ribosomes (A) to read through the uORF (depicted as an orange box) and to initiate translation at the first AUG of the main ORF, generating a long peptide (LP). In contrast, other ribosomes (B) translate the uORF, then resume scanning and may reinitiate translation from the distal AUG of the second ORF to generate a short peptide (SP). TCRS is composed of three cassettes, a 5′ translation regulation cassette containing the *cis*-regulatory uORF, the central peptide expression cassette encoding the LP and SP, and a 3′ regulation cassette, which is devoid of *cis*-elements here. Both peptides harbor an N-terminal pre-pro-trypsin (PPT) sequence for secretion and an HA epitope for immobilization (by sepharose beads or ELISA dishes) and detection purposes. Furthermore, LP and SP can be distinctively identified through the Flag epitope and Myc epitope, respectively. (**b**) Schematic representation of TCRS peptide detection after immobilization on anti-HA–coated solid surface or in solution. (**c**) TCRS and three control constructs with a nonfunctional uORF ($TCRS^{\Delta uORF}$), or encoding solely the LP (TCRS-LP) or SP (TCRS-SP), were cloned into a pSG5-based expression vector for transient transfection into COS-1 cells. Both LP and SP could be detected by immunoblotting using antibodies to (anti-HA). LP and SP could be distinctively detected using anti-Flag and anti-Myc, respectively.

peptides: a long peptide (LP) expressed from a proximal initiation site and a short peptide (SP) expressed from a different reading frame at a distal site. Both peptides start with a pre-pro-trypsin leader sequence, which targets them to the endoplasmic reticulum for subsequent secretion into the culture medium. The peptides contain a common hemagglutinin (HA) epitope, which facilitates immobilization to antibody-coated surfaces for purification and detection purposes (see **Fig. 1**). Discrimination between peptides is accomplished by the unique immunotags Flag and Myc in LP and SP, respectively. Furthermore, eight cysteine residues for ^{35}S radioactive labeling are dispersed throughout each peptide for comparable detection of labeled peptides synthesized using *in vitro* translation systems. The central expression cassette is preceded by a 5′ translation regulation cassette, which contains the *cis*-regulatory uORF of *CEBPA* to control the translation of the two reporter peptides. Finally, putative regulatory sequences may be cloned into the 3′ cassette to examine their effect on translation (not used in this protocol). A negative control construct devoid of a functional uORF ($TCRS^{\Delta uORF}$) is available to distinguish translational control from other regulatory factors and events, such as cryptic promoters, unexpected splicing or mRNA stability[12]. The structure of the TCRS construct and the principles of TCRS peptide detection are depicted in **Figure 1**.

Applications of TCRS

The central idea behind the TCRS design is to provide a rapid and easy-to-handle tool that allows the efficient analysis of translational control processes in the cell. It has a range of applications. For example, compounds may be tested for their putative translational activity using cell lines stably expressing TCRS, or the interrelation between signaling cascades and the translational control machinery may be examined by exposing TCRS-expressing cell lines to the corresponding inducers. Alternatively, TCRS may be transiently transfected into cell lines carrying a gene mutation, to test for a translational response upon aberrant gene expression. Because of the modular design of TCRS, potential upstream *cis*-regulatory elements other than the *CEBPA* uORF may be tested for their putative translational activity. In addition, putative regulator

TABLE 1 | TCRS constructs available cloned into pcDNA3.

Construct	Regulatory sequence	Function	Product
TCRS	uORF of *CEBPA*	Reporter gene	LP peptide (23 kDa) SP peptide (12 kDa)
TCRS$^{\Delta uORF}$	uORF destroyed by ATG→ATC mutation	Negative control Reporter gene function	LP peptide (23 kDa)
TCRS-LP	No	Positive LP expression control	LP peptide (23 kDa)
TCRS-SP	No	Positive SP expression control	SP peptide (12 kDa)

sequences can be cloned in the downstream 3′ cassette for examination of translational activity.

TCRS has the potential to be integrated into ELISA techniques; the common immunotag could be used for immobilization purposes and the distinct second epitope for specific identification of the corresponding peptide. ELISA-based TCRS methods would allow high-throughput screening for the identification of agents modulating translation, and they might have a major impact on the design of novel therapies. The use of TCRS as a transgene in animal models would be useful to study control of translation initiation *in vivo*.

Limitations of TCRS

The TCRS measures efficiency of translation (re)initiation; however, it cannot discriminate between different signal transduction pathways and translation initiation factor(s) that may elicit a change in TCRS response.

MATERIALS

REAGENTS

- TCRS sequence(s) (see **Table 1** and **Fig. 2**) in pcDNA3 (pcDNA3 from Invitrogen); plasmids are shipped upon request to C.F.C.; purchasers are asked to sign a material-transfer agreement form
- Nuclease-free water
- $CaCl_2$ (208291, Merck Biosciences)
- EDTA (324503, Merck Biosciences)
- HEPES, free acid (391340, Merck Biosciences)
- HCl (H1758, Sigma-Aldrich) **! CAUTION** Corrosive: irritating to eyes, respiratory system and skin.
- Methanol (M3641, Sigma-Aldrich) **! CAUTION** Highly flammable. Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed.
- Skim milk powder (70166, Sigma-Aldrich)
- SDS (L4390, Sigma-Aldrich) **! CAUTION** Harmful in contact with skin and if swallowed. Highly flammable.
- NaOH (S8045, Sigma-Aldrich) **! CAUTION** Corrosive: causes severe burns.
- 30% (wt/vol) acrylamide/bis solution (29:1) for SDS-PAGE (161-0156, Bio-Rad) **! CAUTION** Highly toxic: may cause cancer. May cause heritable genetic damage. Possible risk of impaired fertility. Also harmful by inhalation and in contact with skin. Irritating to eyes and skin. May cause sensitization by skin contact.
- DMEM cell culture medium (31966-021, Invitrogen/GIBCO)
- Penicillin-streptomycin solution (15140-122, Invitrogen/GIBCO)
- PVDF membrane Immobilon-P (IPVH00010, Millipore)
- Odyssey blocking buffer (927-40,000, LI-COR Biosciences)
- Roti-Block (Roth)
- Enhanced Chemiluminescence Labeling (ECL) Western Blotting System, containing detection reagents 1 and 2 (RPN2108, GE Healthcare Life Sciences (Amersham))
- ECL anti-mouse IgG HRP (NA931, GE Healthcare Life Sciences (Amersham))
- ECL anti-rabbit IgG HRP (NA934, GE Healthcare Life Sciences (Amersham))
- Mouse anti-FLAG M2 (F3165, Sigma-Aldrich)
- Mouse anti-HA11 (MMS-101R, Covance)
- Mouse anti-Myc (9E10, Santa Cruz Biotechnology, sc-40)
- for Odyssey analysis: anti-mouse IgG Alexa Fluor 680 or IRDye 800 (A21059, Invitrogen)

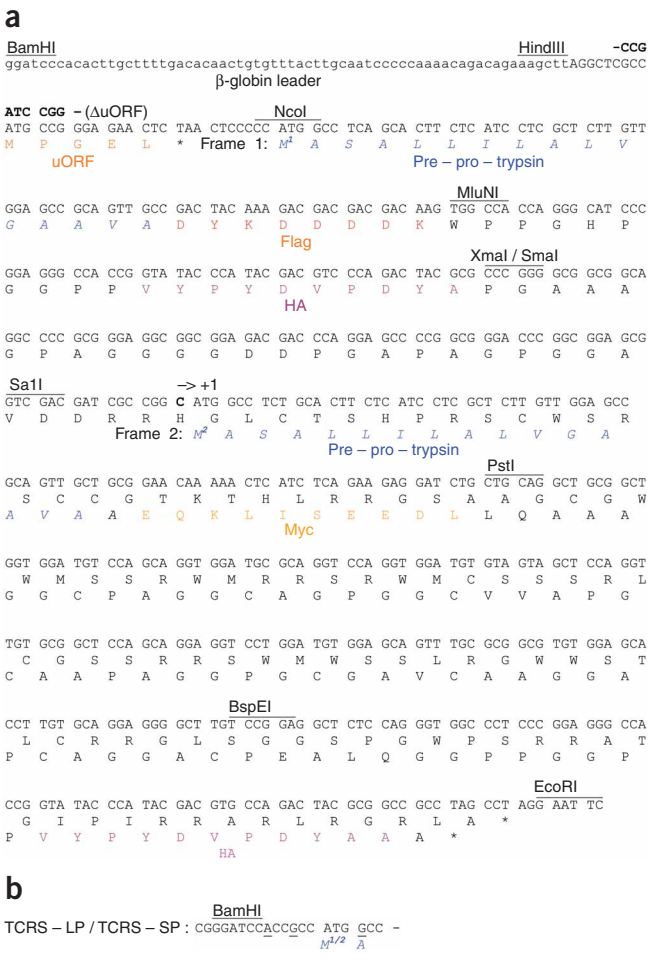

Figure 2 | Nucleotide sequences of the TCRS. All TCRS constructs are flanked on the 5′ side by BamHI and on the 3′ side by EcoRI for cloning into expression plasmids. (**a**) The mutated sequences in TCRS$^{\Delta uORF}$ control are indicated in bold. The unique restriction sites shown may be used for exchange cloning of alternative sequences. (**b**) Shown is the sequence with BamH1 and initiation AUG codon in perfect Kozak consensus (critical nucleotides underlined) of both TCRS-LP and TCRS-SP control constructs. Further sequences are identical to that of TCRS.

EQUIPMENT
- Cell-culture plastics (TPP)
- Cell-culture incubator (37 °C, 5% CO_2, humidified atmosphere)
- Cell-culture centrifuge with swing-out rotor
- Inverted microscope
- Bench-top microfuge, cooled
- Sonopuls HD 2070 ultrasonic homogeniser with microtip MS-72 (Bandelin)
- Heating block
- Standard SDS-PAGE equipment
- Western-blotting equipment
- Rocking table
- Odyssey Infrared System (LI-COR Biosciences)

REAGENT SETUP

2× HEPES-buffered saline 16.4 g NaCl (567441, MERCK Biosciences), 11.9 g HEPES acid, 0.21 g Na_2HPO_4, 800 ml H_2O. Titrate to pH 7.10 with 5 M NaOH. Add H_2O to 1 l. Filter-sterilize through a 0.45-µm nitrocellulose filter. Store at –20 °C in 50-ml aliquots. ▲ CRITICAL Exact pH is crucial for efficient transfection. The optimal pH range is 7.05 to 7.12. Test precipitate formation by adding 0.5 ml $CaCl_2$-water mix (without DNA) dropwise to 0.5 ml 2× HEPES while bubbling. Under the microscope, you should be able to observe evenly distributed very small black particles. ▲ CRITICAL Keep at –20 °C for long-term storage.

TBST 2.42 g Tris base (20 mM; T1503, Sigma-Aldrich), 8 g NaCl (137 mM), 3.8 ml 1 M HCl. Dissolve in H_2O to 1 l total volume. Add 200 µl Tween 20 (655204, Merck Biosciences; final concentration 0.02% (vol/vol)). pH should be 7.6.

PBST 11.5 g Na_2HPO_4 (80 mM), 2.96 g NaH_2PO_4 (20 mM), 5.84 g NaCl (100 mM). Dissolve in H_2O to 1 l total volume. Add 200 µl Tween-20 (0.02% vol/vol). pH should be 7.5.

Milk 5% (wt/vol) skim milk powder dissolved in TBS-T. ▲ CRITICAL should be freshly prepared or thawed from stock kept at –20 °C.

10× SDS electrophoresis buffer 30 g Tris base (0.25 M), 72 g glycine (1.92 M), 10 g SDS (1% wt/vol). Dissolve in H_2O to total volume of 1 l.

10× transfer buffer 24.2 g Tris base (200 mM), 113 g glycine (1.5 M). Dissolve in H_2O to total volume of 1 l. 1× working buffer solution should contain 15% (vol/vol) methanol.

6× SDS loading buffer 1.95 ml 1 M Tris (pH 6.8), 0.9 g SDS, 1.5 ml β-mercaptoethanol (444203, Merck Biosciences), a few crystals bromophenol blue (32768, Sigma-Aldrich). Dissolve in H_2O to total volume of 5 ml. Add 3 ml glycerin. ▲ CRITICAL Stocks should be stored at –20 °C.

PROCEDURE

Constructing appropriate TCRS-containing plasmids ● TIMING 2 d, approximately 3 h each day

1| Use the appropriate restriction sites to individually clone the TCRS sequence and the accompanying negative control ($TCRS^{\Delta uORF}$) and positive controls (TCRS-LP and TCRS-SP) into any appropriate expression vector; all cassettes are flanked by a BamHI restriction site at the 5′ terminus and an EcoRI restriction site at the 3′ terminus (see **Fig. 2**). Standard cloning procedures can be used[3]. We have used pcDNA3-based expression plasmids for stable transfection of C33A and HEK293A cells and pSG5-based expression plasmids for transient expression in COS-1 cells[12].

▲ CRITICAL STEP This protocol uses the TCRS as described in **Figure 2**. Please note that TCRS has a modular structure allowing each module, immunoepitope, 5′ UTR and 3′ UTR to be replaced by sequences of your choice. The unique cloning sites flanking each module can be used to exchange it by standard cloning techniques[3].

Transient and stable transfection with TCRS ● TIMING 6–7 d for transient transfection; up to 14 d for stable transfection

2| Seed cells at a density of approximately 4×10^6 cells per 10-cm dish and incubate at 37 °C overnight in a CO_2 cell-culture incubator. The cells should be well separated the next day. Thus far, we have tested the transient and stable transfection protocols only on adherent cells; different transfection protocols may be required for cells propagated in suspension.

▲ CRITICAL STEP The amount to be seeded may vary depending on the growth rate and size of the cells. Cells should be solitary at the time of transfection to enhance efficiency of transfection and to allow convenient selection in growth medium supplemented with selective antibiotics.

3| Dissolve 5–10 µg of plasmid DNA in 450 µl sterile nuclease-free water in a 10-ml conical polystyrene tube and add 50 µl of filter-sterilized 2.5 M $CaCl_2$.

4| Bubble the DNA-$CaCl_2$ solution vigorously with a plugged sterile Pasteur pipette and an automatic pipettor and add 500 µl of filter-sterilized 2× HEPES dropwise. ▲ CRITICAL STEP HEPES buffer is the critical parameter for successful transfections. Test precipitate formation by adding 0.5 ml $CaCl_2$-water mix (without DNA) dropwise to 0.5 ml 2× HEPES while bubbling. Under the microscope, you should be able to observe evenly distributed very small black particles.

? TROUBLESHOOTING

5| Vortex the transfection mix briefly (1 s) but vigorously, and immediately disperse the solution by dropwise addition to the cells. Gently agitate to ensure an even distribution of the DNA precipitate.

6| Incubate at 37 °C overnight in a CO_2 cell-culture incubator.

7| The next morning, aspirate the medium, wash the cells twice with PBS, replace with fresh medium and incubate at 37 °C for an additional 24 h.

▲ CRITICAL STEP Failure to remove precipitates properly may be toxic to the cells. Additional washing with PBS may be required if residual precipitates are visible under the microscope.

8| Use the cells for transient transfection assays (A) or to establish a stable cell line (B).

(A) For immediate assays of transiently transfected cells

(i) Cultivate the cells using the conditions and medium appropriate to the cell type being used.

(ii) Harvest the cells 24 to 48 h after transfection (see Step 7).

(B) To generate a stable TCRS-expressing cell line

(i) Select for integration of the TCRS sequence into the genome by adding the appropriate selective agent to the growth medium.
▲ CRITICAL STEP The appropriate selective compound needs to be matched to the selective marker in the expression vector—for example, G418 for pcDNA3-based plasmids. The concentration of each compound needs to be adjusted to each cell type.

(ii) Grow the cells in appropriate conditions in selective medium for 3 to 8 d, depending on the selective agent, until single colonies become visible; these are cells that have stably integrated the TCRS expression vector DNA into their genome.

(iii) At this stage, either isolate and further propagate single colonies to establish stable cell lines[3] or pool colonies for further experiments, which is sufficient for most purposes.
▲ CRITICAL STEP Low-passage cells should be frozen at this stage. Upon thawing, cells need to be maintained under selective conditions. If cells are close to confluence, selection by some agents may be unsuccessful. In this case, cells need to be split 1:10 to 1:20 to allow efficient selection.
? TROUBLESHOOTING

Example experiment: treatment with the mTOR inhibitor rapamycin ● TIMING 5 d

9| Seed 12 culture dishes with stable TCRS-expressing cells (see **Fig. 3**; HEK293A-TCRS cells) and cultivate until confluent. Apply 1 μM rapamycin to 6 dishes and solvent only to the other 6 dishes as a negative control. Harvest and prepare cell extracts after 0, 2, 4, 8, 12 and 24 h treatment as described below.

Preparation of total cell lysates (after experimental treatment of TCRS-expressing cells) ● TIMING 1–2 h

10| Aspirate medium and wash once with ice-cold PBS.

11| Add 1 ml of PBS to each dish and harvest the cells by scraping.

12| Transfer the cell suspension into a 1.5-ml microfuge tube and pellet by centrifugation for 1 min at 12,000*g* to 14,000*g* at 4 °C and discard supernatant.
■ PAUSE POINT At this stage, cell pellets may be snap-frozen in liquid nitrogen and stored at −80 °C.

13| To rupture ~5 × 10^6 cells, add 30 μl of 0.5 M NaOH and try to resuspend the pellet by vigorous vortexing for approximately 30 s, until cell pellet is completely dissolved.

14| Immediately neutralize the sample through the addition of 30 μl 0.5 M HCl.

15| Add 16 μl SDS loading buffer (see REAGENT SETUP) and mix by vortexing for approximately 30 s.
▲ CRITICAL STEP The sample should now appear blue. A yellow color indicates an acid environment, and more 0.5 M NaOH should be added dropwise.

16| Homogenise the cell extract by sonification using an ultrasonic rod-homogeniser.
▲ CRITICAL STEP After sonification, the lysate should be fluid; a viscous appearance indicates incomplete rupture of cells, and sonification should be repeated.

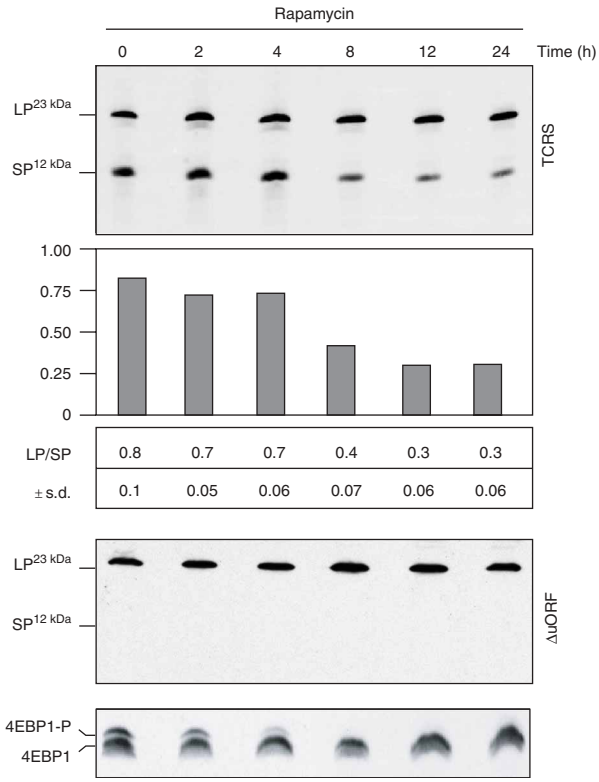

Figure 3 | Measurement of rapamycin-induced attenuation of translation by TCRS in HEK293A cells. Rapamycin (1 μM) inhibits mTOR signaling and attenuates translation through the hypophosphorylation of 4E-BP1 (bottom blot; 4E-BP1-P, phosphorylated protein) and subsequent sequestering of eIF4E, which is indicated by a decrease in SP expression. The SP/LP ratio was quantified using fluorescent dye–conjugated secondary antibodies to HA and the Odyssey Infrared Imaging System (LI-COR). Bar graph shows change in SP/LP ratio upon rapamycin treatment, derived from three independent experiments. SP expression was not detected from the control construct lacking the *cis*-regulatory uORF ($TCRS^{\Delta uORF}$, middle gel).

17| Heat the sonicated sample to 98 °C for 3 min, then briefly centrifuge to collect all extract at the bottom of the tube.
■ **PAUSE POINT** Samples may be frozen at −20 °C.
▲ **CRITICAL STEP** If the cell extract is to be loaded immediately onto the SDS gel, do not put sample on ice after heating, as the SDS in the loading buffer may precipitate.

Analysis of TCRS expression profile using immuno-blots. ● **TIMING 1 d (~8 h)**
18| Separate proteins by standard SDS-PAGE[3]. Load 5% to 10% of the total volume of cell lysates on a 15% SDS polyacrylamide gel and allow to run until the blue dye has reached the bottom of the gel.
▲ **CRITICAL STEP** Because of small TCRS peptide sizes (LP=23 kDa; SP=12 kDa) a high-percentage gel is required for optimal resolution.

19| Activate the PVDF membrane by submerging in methanol for 1 min, rinsing in water for 2 min and soaking in transfer buffer (see REAGENT SETUP) for 5 min.

20| Set up the electrophoretic transfer. Perform transfer of proteins at 80 V for 1 h in a cold room (4 °C).
▲ **CRITICAL STEP** Blotting efficiency may vary considerably depending on the blotting equipment and conditions. Optimization may be required[3].

21| Dissemble blotting equipment and immerse membrane in blocking buffer for 30 min to saturate the protein binding sites. Blocking buffers may be detergent-based solutions such as Roti-Block or LI-COR block (best for later analysis by the LI-COR Odyssey) or 5% dry milk in TBST or PBST (for ECL analysis).
■ **PAUSE POINT** Membranes can be stored a few weeks in TBST or PBST at 4 °C.

22| Incubate the membrane with primary antibody at optimal titer (as recommended by manufacturer) in the appropriate blocking buffer, for 1 h (at room temperature, 20–25 °C) to overnight (at 4 °C) under gentle shaking on a rocking table. An antibody to HA (1:1,000) will detect both LP and SP; anti-Flag (1:450) will exclusively detect LP; and anti-Myc (1:1,000) will specifically detect SP.
▲ **CRITICAL STEP** The optimal antibody concentration may have to be determined, per manufacturer's recommendations.

23| Wash membrane three times for 5 min and twice for 10 min with TBST (or PBST).

24| Apply the secondary antibody (goat anti-mouse Alex-flour 680 nm for the Odyssey in Roti-Block or LI-COR block at a 1:2,500 dilution, or anti-mouse IgG HRP for the ECL system in milk at a 1:5,000 dilution) and incubate for 1 h at room temperature.
▲ **CRITICAL STEP** To retain optimal performance of the fluorescence-labeled secondary LI-COR antibody, this step should be performed in the dark.

25| Wash membrane again three times for 5 min and twice for 10 min with TBST (or PBST).

26| Visualize the proteins using either LI-COR Odyssey (A) or ECL (B) detection.
(A) Li-COR Odyssey
(i) The membrane is ready to be directly scanned and analyzed using the Odyssey software. Signals on one blot can be compared quantitatively using the Odyssey software.
(B) For chemiluminescent (ECL) detection
(i) Drain off washing buffer.
(ii) Add equal volumes of ECL detection reagent 1 (oxidant-containing buffer) and ECL detection reagent 2 (luminol substrate buffer), sufficient to cover the membrane, and incubate for 1 min.
(iii) Wrap membrane in cling film (saran wrap) immediately, and expose to autoradiography film initially for 1 to 10 min using a standard film cassette. If the signal is weak when the film is developed, replace the film and expose for a longer time period. The signals on the film may be quantified by scanning and analysis using imaging software.
▲ **CRITICAL** The chemiluminescence (ECL) signal is of dynamic enzymatic origin. The signal on the film and thus the quantification is highly dependent on start time and exposure duration. The Odyssey system or other comparable systems are the systems of choice, if available.
■ **PAUSE POINT** Blots can be retained for several weeks in PBST or TBST at 4 °C. Blots visualised with fluorescently labeled antibodies in Step 25A should be kept in the dark.
? TROUBLESHOOTING

● **TIMING**
Step 1: day 0 to 2, approximately 3 h each day
Steps 2–8: days 3–9 (or 17). For transient transfection, approximately 1 h per day for 6–7 d; for stable transfection, 1 h per day for up to 14 d

Step 9: 5 d. 1 h at day 1 for seeding the cells, whole day 3 for rapamycin treatment, 1 h at day 4
Steps 10–17: 1–2 h
Steps 18–26: 1 d (~8 h)

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Solution
Step 4, no precipitates	Recheck the pH of the 2× HEPES, which should be at 7.1 (7.05–7.12 is acceptable). In some laboratories, acidity of water may prevent formation of fine precipitates. In such cases, a $T_{10}E_1$ (10 mM Tris-HCl, 1 mM EDTA[3]) pH 7.05 solution should be used to dissolve the DNA.
Step 8B, all cells die	Transfection failed; repeat the transfection. Concentration of selective agent too high; retry with lower concentrations of 3:4 and 1:2.
Step 8B, all cells survive	Concentration of selective agent too low; increase the concentration.
Step 26, no TCRS peptide detected	To exclude technical reasons (for example, failure of blotting, ECL reaction or antibody interaction), a control extract with previously detectable HA-, Flag- or Myc-tagged peptide(s) can be loaded together with the TCRS.

ANTICIPATED RESULTS

Typically, two bands, representing the TCRS-LP (23 kDa) and the TCRS-SP (12 kDa), can be distinguished on an anti-HA–probed western blot (**Fig. 3**). These bands can be quantified, which may reveal a shift in the SP/LP ratio upon exposure to a translationally active drug such as, in this case, rapamycin. As an example, the mammalian target of rapamycin (mTOR) stimulates eukaryotic translation initiation factor 4E (eIF4E)-mediated initiation of translation by maintaining eIF4E-binding proteins (4E-BPs) in a hyperphosphorylated and inactive state[13]. Rapamycin inhibits the mTOR kinase and thereby attenuates translation. For this experiment, 1×10^7 TCRS-HEK293A cells (in a 10-cm dish) were exposed to 1 μM rapamycin and harvested after 2, 4, 8, 12 and 24 h. One tenth of the corresponding lysates were separated by SDS-PAGE (15%) and electroblotted as described in the protocol. The inhibitory effect of 1 mM rapamycin on translation initiation can be monitored by TCRS (**Fig. 3**). Concomitant with the abolishment of phosphorylated 4E-BPs after 8 h of treatment (**Fig. 3**, bottom blot), a two-fold decrease in SP expression was measured, and expression gradually decreases even further (~2.6-fold) after 24 h of treatment. Elimination of the uORF ($TCRS^{\Delta uORF}$) entirely abolishes SP expression, irrespective of external stimulation.

ACKNOWLEDGMENTS This work was supported by a Max Delbrück Centre grant to V.W. and a Deutsche Forschungsgemeinschaft grant to A.L. and C.F.C. (LE 770/3-2).

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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