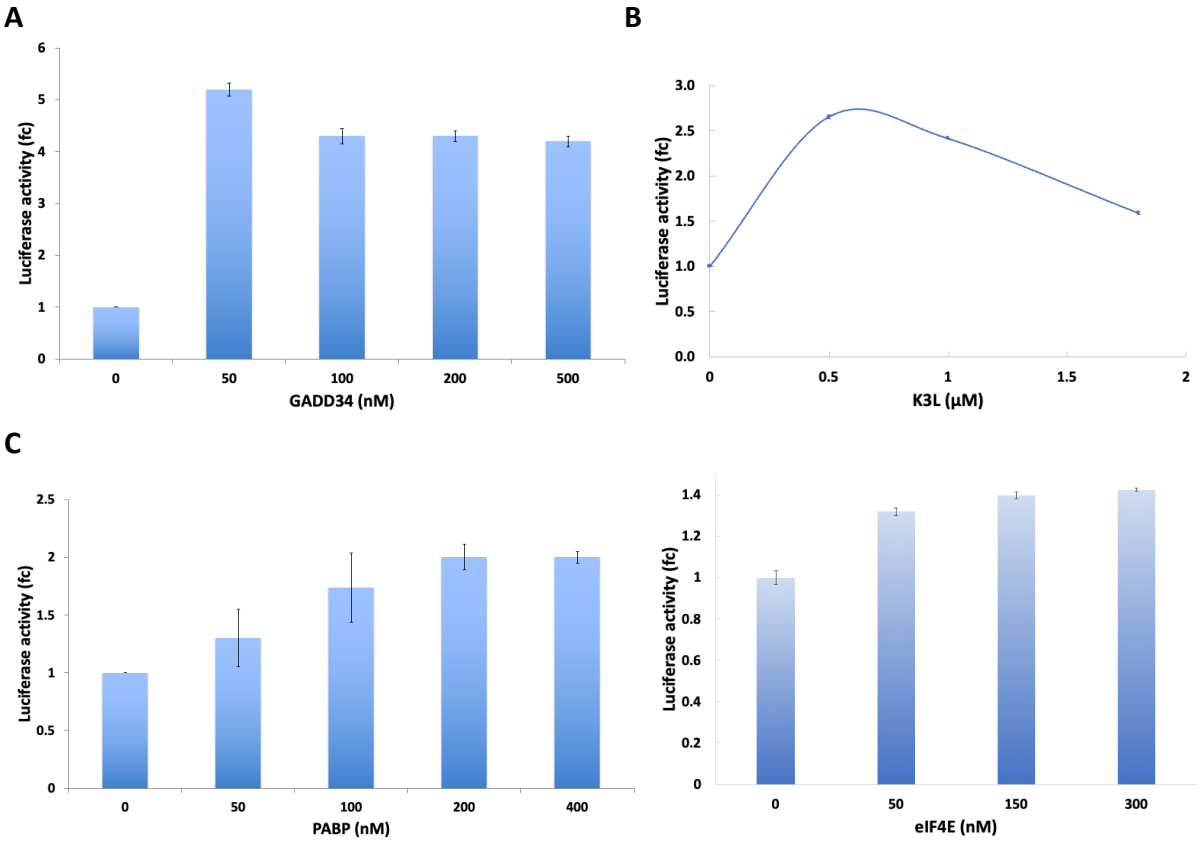
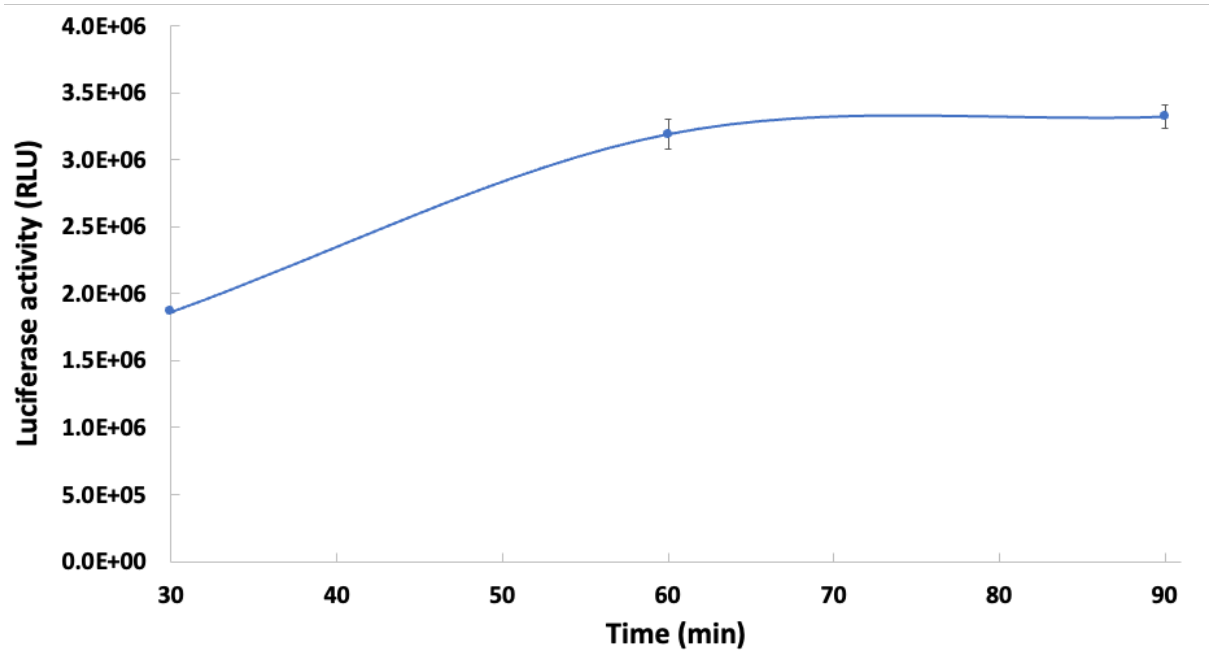
Supplemental Figures



Supplemental Figure S1



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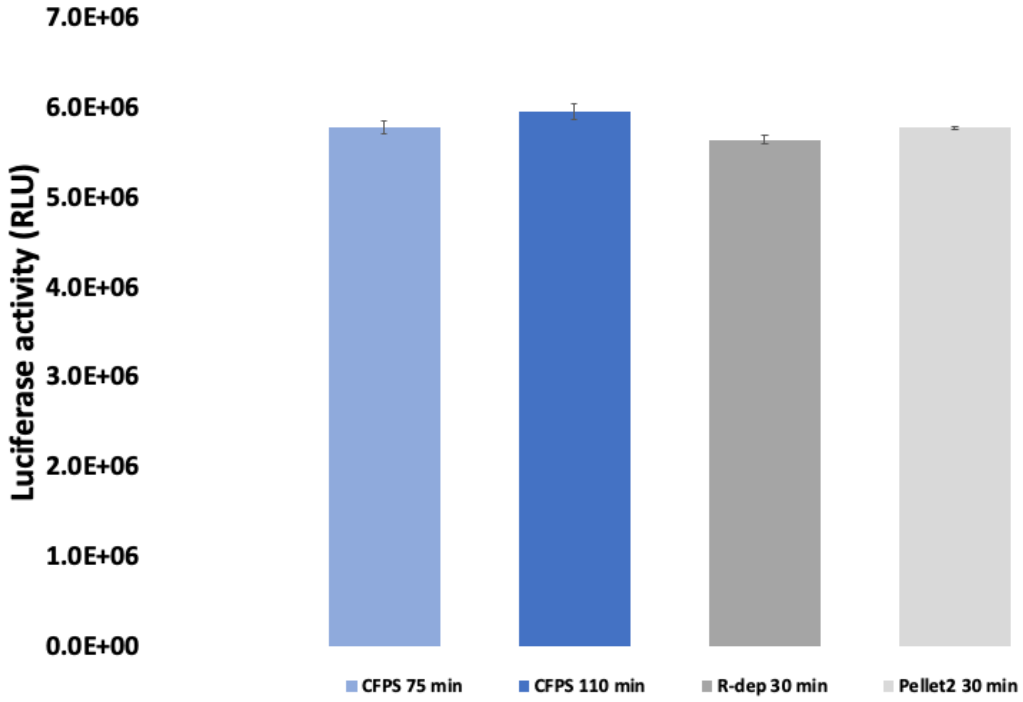
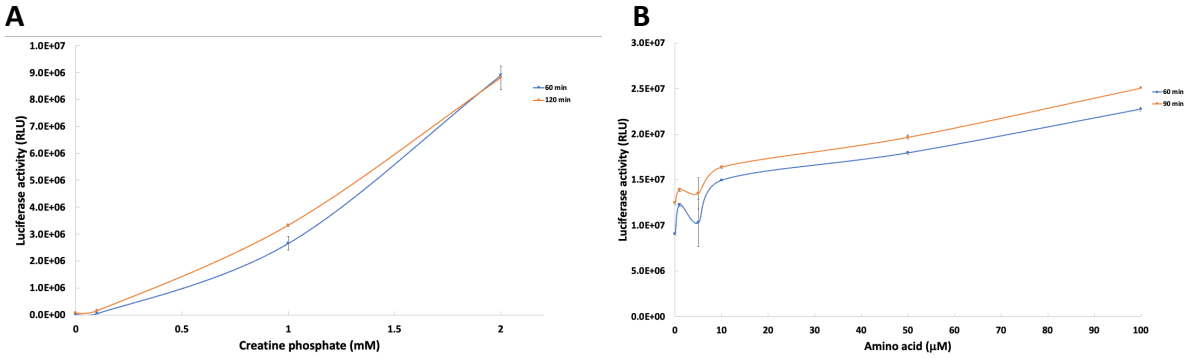
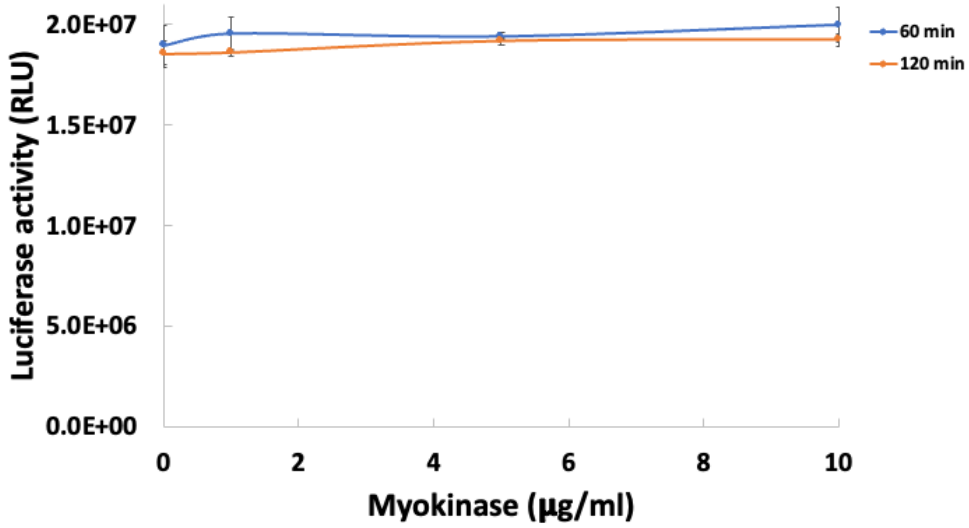


Supplemental Figure S3.



Supplemental Figure S4.



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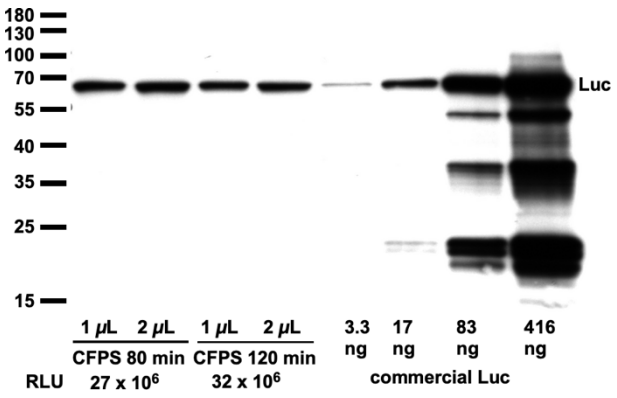
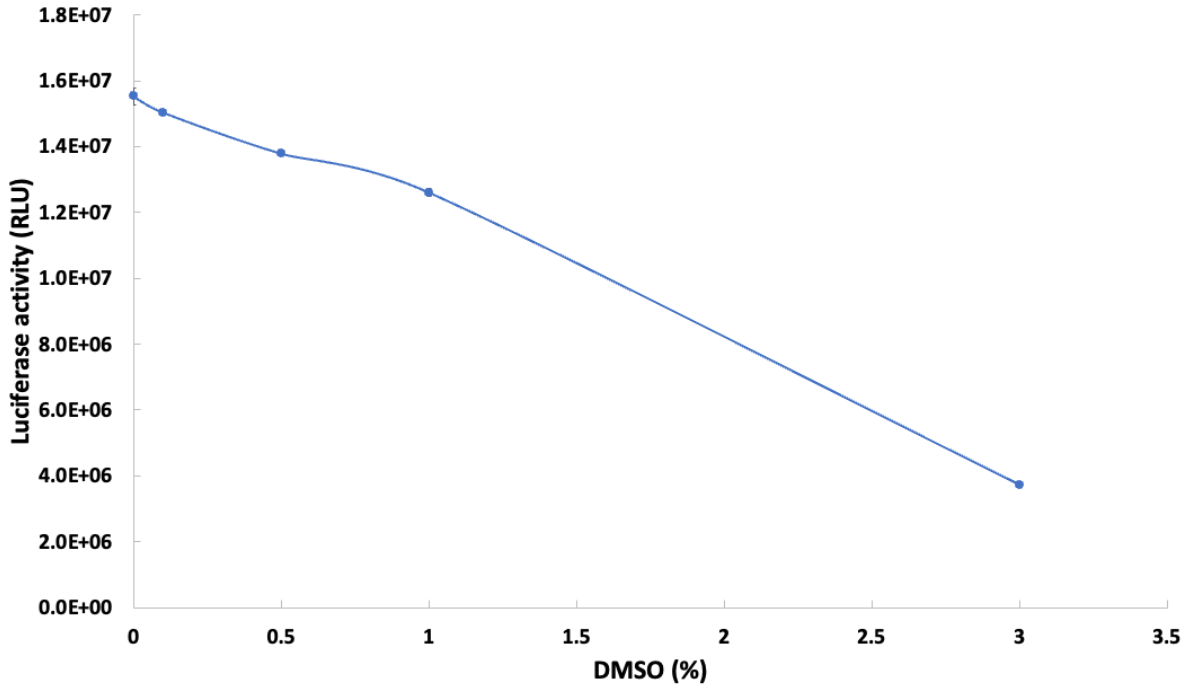


Supplemental Figure S6.



Supplemental Figure S7.

Supplemental Figure S1. Exogenously added proteins GADD34 and K3L increase the protein synthesis activity of the HEK293-based CFPS system. (A) GADD34 concentration (0 to 500 nM) was titrated in CFPS assay programmed with capped IFITM1-Luc mRNA. Synthesized reporter luciferase activity was monitored using Steady-Glo (Promega) reporter assay. Luciferase activity (fc: fold change compared to 0 nM GADD34) increases by approximately 5-fold with the addition of 50 nM GADD34. The CFPS reaction was incubated 30 min at 30° C. The data represent the average of three experiments with the standard deviation from the mean. (B) Adding the recombinant protein K3L to the CFPS reaction at a concentration of 0.5 µM increases luciferase activity by 2.7-fold compared to the reaction with no added K3L. The CFPS reaction was incubated 30 min at 30° C. The data represent the average of three experiments with the standard deviation from the mean. (C) PABP addition to the CFPS increases luciferase activity by 2-fold compared to the reaction with no added PABP. The CFPS reaction was incubated 30 min at 30° C. The data represent the average of four experiments with the standard deviation from the mean. (D) eIF4E addition to the CFPS increases luciferase activity slightly by 1.4-fold compared to the reaction with no added eIF4E. The CFPS reaction was incubated 2h at 30° C. The data represent the average of two experiments with the standard deviation from the mean.

Supplemental Figure S2. The human neuroblastoma cell line SH-SY5Y-based CFPS exhibits a similar time-dependent inhibition as the HEK293-based CFPS. The CFPS system was programmed with capped IFITM-Luc mRNA and luciferase activity was monitored for 90 min. The data represent the average of two experiments with the standard deviation from the mean.

Supplemental Figure S3. Adding R-dep or Pellet2 to already inhibited HEK293 CFPS reaction. The CFPS reaction of HEK293 was incubated at 30°C until CFPS ceased (CFPS 110 min) after which R-dep (R-dep 30 min) or Pellet2 (Pellet2 30 min) were added to the inhibited CFPS reaction and incubated additional 30 min at 30°C. If ribosomes (Pellet2) or translation factors (R-dep) were the cause of the inhibition, then adding fresh ones back would activate the already inhibited components. However, no CFPS activation is observed when Pellet2 or R-dep is added to the inhibited CFPS reaction. The data represent the average of two experiments with the standard deviation from the mean.

Supplemental Figure S4. Native creatine kinases of HEK293 extract are active in the CFPS. (A) The ATP was omitted from the CFPS reaction, and the range of 0.1 to 2 mM creatine phosphate concentration was titrated. Without ATP and CrP, the CFPS is inactive. Increasing the CrP concentration activates the CFPS, indicating that native CK's are actively regenerating ATP from CrP. Two time points were monitored, 60 min (blue) and 120 min (orange). The data represent the average of two experiments with the standard deviation from the mean. (B) The amino acids concentration (0 to 100 µM) was titrated in the CFPS assay. The CFPS is active even without the addition of amino acids although 2-fold lesser activity is observed compared to 100 µM amino acids. The data represent the average of two experiments with the standard deviation from the mean.

Supplemental Figure S5. Rabbit myokinase does not increase the CFPS activity in HEK293 extract. Indicated concentration of rabbit myokinase were added to the CFPS reaction and CFPS activity was monitored at 60 min (blue) and 120 min (orange) time intervals. The data represent the average of two experiments with the standard deviation from the mean.

Supplemental Figure S6. Estimation of luciferase produced in the CFPS assay using Western blot analysis. The CFPS reaction (2 mM CrP was used) 1 µl or 2 µl was loaded to protein SDS-gel. The RLU activity measured from 3 µl reaction is indicated below. The commercial luciferase with known concentration was used as reference. Left lane shows the protein marker lines in kDa that were added to the image file using CanvasX software otherwise original gels are presented.

Supplemental Figure S7. Testing the organic solvent (DMSO) effect to CFPS. DMSO concentration up to 5% was used in the CFPS reaction and CFPS activity was measured after 60 min incubation. DMSO decreases the CFPS activity in concentration dependent manner, where 5% DMSO reduced the CFPS activity by 4-fold. The data represent the average of two experiments with the standard deviation from the mean.

Supplement Materials and Methods

Treating the CFPS reaction with Pellet2 or R-dep. The CFPS reaction was set up as described above and incubated at 30° C for 110 min. After incubation, 17 µl of CFPS reaction was taken to new tube and 2 µl of MNase treated Pellet2 or R-dep was added. The reactions were incubated additional 30 min at 30° C and luciferase activity was measured as above.

DNA constructs

The K3L, GADD34, and PABP DNA coding sequences were inserted into Addgene, pET His6 MBP Asn10 TEV LIC, 1C vector using Lic cloning protocol. The DNA sequences were codon optimized for E. coli

expression using IDT codon optimization tool. The gBlock fragments for each protein were ordered from IDT.

PABP primers used for Lic cloning:
PABP-f: TACTTCCAATCCAATGCAAACCCGAGCGCACCGAGTTACC
PABP-r: TTATCCACTTCCAATGTTATTATCAAACCGTCGGCACACCCGTG
GADD34 primers used for Lic cloning:
Gad-f: TACTTCCAATCCAATGCAAAAGGAGCCAGGAAGACCTCCGTG
Gad-r: TTATCCACTTCCAATGTTATTATCAGCCACGCCTCCCACTGAGGTCC
K3L primers used for Lic cloning:
K3L-f: TACTTCCAATCCAATGCATTAGCCTTTTGTTATTCGCTC
K3L-r: TTATCCACTTCCAATGTTATTATCACTGGTGGCGACACATACG

PABP gBlock sequence:
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N-terminal deletion of 240 amino acids from GADD34 gBlock sequence:
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K3L gBlock sequence:
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DNA constructs for mRNA
gBlock fragments for mRNA constructs (IFITM1-Luc, EMCV-IRES-Luc, CrPV-IRES-Luc, HCV-IRES-Luc) were ordered from IDT. The gBlock fragments were PCR amplified using specific primers for each construct and inserted into the pUC19 vector into the HindIII and BamHI site (IFITM1-Luc, CrPV-IRES-Luc, HCV-IRES-Luc) or SalI and BamHI site (EMCV-IRES-Luc).
Primers used for cloning mRNA constructs into pUC19 vector
Crvp-f: ATTTCAAGCTTTAATACGACTCACTATAG
Crvp-r: AAATGGATCCTTATTACAATTTGGACTTTC
Ifitm-f: ATTTCAAGCTTTAATACGACTCACTATAGGAAACG
Ifitm-r: ATTTGGATCCTCACAAGCACGTGCAC
Emcv-f: ATTTCGTCGACTAATACGACTCACTATAGGTAACGTTACTGGCCGAAGC
Emcv-r: ATTTCGGATCCTTACAATTTGGACTTTCCGCC
HCV was cloned using Crvp primers
Primers used for PCR amplification and addition of poly(A)-tail of mRNA constructs
CrPV
Crvp-f: ATTTCAAGCTTTAATACGACTCACTATAG
Luc-pA70:
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HCV
Crvp-f: ATTTCAAGCTTTAATACGACTCACTATAG
Luc-pA70:
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HCV-IRES sequence is underlined

EMCV-IRES-Luc gBlock sequence:

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EMCV-IRES sequence is underlined

IFITM1-Luc gBlock sequence:

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CACTCCACTGTGCAATGCTGGCCCTGCACGCTGGGGCTGTTGCCCCTGCCCCCTTGGTCCTGCCCCTAGATACAG
CAGTTTATACCCACACACCTGTCTACAGTGTCATTCAATA AAGTGCACGTGCTTGTGA GGATCCATTT

Underlined are the IFITM1 5'- and 3'-UTR sequences. IFITM1 sequence was deduced from NCBI Reference Sequence: NM_003641.5

Protein Purification

K3L, GADD34, and PABP were expressed using a pET-derived expression vector (Addgene, pET His6 MBP Asn10 TEV LIC, 1C) in *E. coli* Rosetta strain as N-terminal fusions to an N-terminally His_6-tagged *E.coli* maltose-binding protein (MBP). eIF4E plasmid was kind gift from Christopher S. Fraser lab and eIF4E was purified according to a previously published procedure (Feoktistova et al. 2013). For protein expression, cells were grown in 1 L 2 × YT medium at 37° C under constant shaking at 140 rpm. Upon reaching a mid-log phase (OD_{600} ≈ 0.6) IPTG was added to the cultures to an f.c. of 1 mM and the incubation continued for 3 h. Cells were harvested by centrifugation in a Herolab A6.9 rotor at 2000 × g/4 C for 10 min. The supernatant was decanted and the cell pellet flash-frozen in liquid nitrogen and stored at – 80 C until further processing. The cells were thawed on ice and resuspended in 35 mL ice-cold lysis buffer for GADD34 and K3L (40 mM Hepes-KOH, pH 7.5, 600 mM KCl, 15 mM imidazole, 2 mM beta-mercaptoethanol β-Me) for PABP (40 mM Hepes-KOH, pH 7.5, 500 mM KCl, 10 mM imidazole, 2 mM β-Me) in the presence of 0.1 mM PMSF and a protease inhibitor cocktail (cOmplete, EDTA-free; Roche). Triton X-100 (f.c. 0.1 % (v/v)), lyzozyme (f.c. 1 mg/mL) and DNase I (f.c 43 U/mL) were added to the suspension and the suspension was incubated at 4° C on an end-over-end shaker for 15 min. The cells were subsequently lyzed by sonication in a Bandelin Sonoplus sonicator at 55 % power through 10 cycles (10 s each) on ice and the lysate was clarified by centrifugation in a Sorval SS-34 rotor at 34 540 × g/4° C for 40 min. The lysate supernatant was additionally filtered through a 0.22 µm syringe filter. All subsequent chromatographic procedures were performed at 4° C.

The clarified lysate was applied to a Ni-Sepharose affinity column (HisTrap 5 ml; Cytiva) at a flow rate of 1 mL/min using a peristaltic pump. After washing the column with 3 column volumes of HisTrap buffer [20 mM Hepes, pH 7.5, 0.8 M KCl (for PABP)/0.5 M KCl (for K3L and GADD34, 20 mM imidazole, 2 mM β-Me] at a flow rate of 1 mL/min, the column was connected to a GE Healthcare ÄKTA Purifier system and washed with additional 3 column volumes of HisTrap buffer at a flow rate of 1 mL/min. Bound protein was eluted from the Ni-Sepharose column using a linear gradient of imidazole from 20 to 300 mM, HisTrap B buffer [20 mM Hepes, pH 7.5, 0.8 M KCl (for PABP)/1 M KCl (for K3L and GADD34), 300 mM imidazole, 2 mM β-Me] at a flow rate of 2 mL/min. Fractions corresponding to the major peak were pooled and applied to an MBP affinity column (MBPTrap 10 ml; Cytiva) at a flow rate of 2 mL/min. After washing the MBP column with 3 column volumes of MBP buffer [20 mM Hepes, pH 7.5, 0.6 M KCl (for PABP and K3L)/0.5 M KCl (for GADD34), 10 % glycerol (for K3L)/ 5 % glycerol (for PABP), 2 mM beta-mercaptoethanol], bound proteins were eluted with 10 mM D-maltose in MBP buffer at a flow

rate of 2 mL/min. The eluted fractions were pooled and incubated with TEV protease at an enzyme-to-substrate ratio ranging from 1 : 100 to 1 :150 at 4^{o} C overnight on an end-over-end shaker.
The K3L protein sample was fractionated on a HiLoad 26/600 Superdex 75 (Cytiva) size exclusion column in S75 buffer (20 mM HEPES, pH7.5, 200 mM KCl, 10% glycerol, and 2 mM β-Me. K3L fractions were pooled and concentrated with Vivaspin Turbo 15 ml, 3000 MWCO filtration unit.
The TEV cleaved GADD34 sample was loaded on the Ni-Sepharose column and eluted at linear gradient 20 mM to 250 mM imidazole in HisTrap buffer at a flow rate of 2 mL/min. Under those conditions the non-tagged GADD34 elute around 30 % to 50 % buffer B and His_6-MBP around 65 % to 88 %. The GADD34 fractions were pooled, concentrated and buffer exchanged to 20 mM HEPES, pH7.5, 200 mM KCl, 10% glycerol, 1 mM TCEP with 10 kDa MWCO Amicon ULTRA-4 (Millipor).
The TEV cleaved PABP sample was buffer exchanged to SP100 buffer (20 mM HEPES, pH7.5, 100 mM KCl, 2 mM β-Me) using a HiPrep 26/10 desalting column (Cytiva). The buffer exchanged PABP sample was loaded on a cation exchange HiScreen SP HP column. The PABP was eluted with a 100 mM – 500 mM KCl gradient at a flow-rate of 0.5 mL/min. The final purification step for PABP was the size exclusion chromatography using the HiPrep Sephacryl 200 column (Cytiva). The column was equilibrated with SP200 buffer (20 mM HEPES, pH7.5, 200 mM KCl, 2 mM β-Me). The eluted PABP was concentrated with 30 kDa MWCO Amicon ULTRA-15 (Cytiva) and final protein buffer composition was 20 mM HEPES, pH7.5, 200 mM KCl, 10 % glycerol, 1 mM TCEP.
All protein concentrations were measured via 10-fold dilutions in 6 M GuHCl using known extinction coefficients. Before storage, all proteins were aliquoted and flash-frozen in liquid nitrogen.