Supplementary information

Legends of movies: Sup_1.mpg; Sup_2.mpg and Sup_3.mpg.

Thirty-two hours after transfection, a cover glass on which transfected cells were grown was assembled into a LUDIN chamber (Life Imaging Services, Switzerland). Time-lapse microscopy of cells expressing hnRNP C1-GFP and PTB-HcRed was carried out using a Leica AS MDW live cell imaging system (Leica Microsystems, Mannheim, Germany). This system includes a DM IRE2 microscope equipped with an HCX PL APO 63x/1.3 glycerin-corrected 37°C objective and a 12 bit Coolsnap HQ Camera. The microscope is equipped with an incubation chamber, which keeps the cells at 37°C in a 5% CO₂ environment. Differential interference contrast and fluorescence images were taken every 2.5 minutes for 16 hours long at 10 z-positions. The GFP and HcRed fluorescence images were monitored using a monochromator system with a 75Watt Xenon lamp for excitation in combination with a standard B/G/R filtercube. Resolution of the images was enhanced using the Huygens deconvolution software (Scientific Volume Imaging BV, Hilversum, The Netherlands). DIC/ GFP / HcRed overlay images were sequentially assembled as a movie (Sup_1_time lapse.mpg). For timepoints 37:30 and 40:00 3D rendering was performed and assemble as movie (Sup_2_3Da.mpg and Sup_3_3Db.mpg).

Materials and methods

Construction of plasmids

The bicistronic pSV-Sport reporter vectors Di-pRF and Di-pFR containing UNR, UNR (1-261), UNR (1-334), UNR (1-390) or UNR (Δ335-355) IRES fragments inserted between the Renilla (R) and firefly (F) luciferase (luc) genes have been described previously (Cornelis et al., 2005). The deletion mutant UNR (261-447) was amplified by PCR with primer pair A+B (see below). The deletion mutants UNR (Δ255-270) and UNR (Δ348-355) were constructed by overlap PCR reactions using primers C+D and E+F, respectively, as overlap primers. The corresponding bicistronic pSV-Sport expression vectors Di-pRF-UNR (261-447) Di-pRF-UNR (Δ255-270) and Di-pRF-UNR (Δ348-355) were obtained by two consecutive three-point ligations as follows: i) UNR fragments digested with XbaI-NcoI were cloned together with the firefly luciferase gene, obtained as an NcoI-HindIII fragment from pSV-Sport-Fluc, in the
XbaI-HindIII linearized vector pUC19; ii) the UNR-Fluc inserts were then recovered as XbaI fragments and cloned in the XbaI linearized pSV-Sport-Rluc.

For the construction of the bicistronic PITSLRE D4 and PITSLRE D4 mut.B reporter vectors see Tinton et al., 2005.

The human hnRNP C1 coding sequence was isolated by a 5’-RACE reaction on poly(A)+ mRNA from HeLa cells, using the SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s instructions. The hnRNP C1 coding sequence was amplified using primer pair G+H and fused to a Flag-tag as a KpnI/XhoI fragment in the pCAGGS expression vector.

The UNR cDNA (a kind gift from Dr. Jackson) was amplified using primer pair I+J, and fused to an E-tag as a NotI/XhoI fragment in the pCAGGS expression vector.

In order to make the pPTB-HcRED expression vector, we first constructed the pPTB-EGFP vector by cloning the PTB cDNA (a kind gift from Dr. Jackson), amplified by primer pair K+L, in a BamHI/EcoRI opened pEGFP-N3 vector (BD, Clontech). The HcRed coding sequence was amplified from the pHcRed1 (BD, Clontech) by primer pair M+N. Finally, the EGFP coding sequence was removed from the pPTB-EGFP vector by digestion with BamHI and HpaI and replaced by the BamHI/HpaI digested HcRed fragment, to yield pPTB-HcRED vector.

To construct the pEGFP-hnRNPC1 expression vector, the hnRNPC1 cDNA was amplified using primer pair O+P. The amplified product was cloned in a KpnI/BamHI opened pEGFP-C1 vector (BD, Clontech).

For the UV crosslinking and RNA affinity chromatography experiments, the different UNR PCR fragments and PITSLRE IRES fragments (XbaI-NcoI digested) were cloned into the pUC19 plasmid in which a T7 promoter had been inserted (Tinton et al., 2005).

The following primers were used:

A, 5´-CTAGTCTAGATGTTTTTCTTCAGTGCTACTGTGAGATTGCC -3´;  
B, 5´-CATGCCATGGCGCAGTGATCTAAATATTGCACTTTTCAGT -3´;  
C, 5´-TACTAGCAGCGTTGGAAATGTC -3´;  
D, 5´-GAGAGAAAATGATCTACCAAGCTAA-3´;  
E, 5´-GAGAGAAAATGATCTACCAAGCTAA-3´;  
F, 5´-GCTTGGTAGATCATTTTCTCTCAATTAGACTGAACCTCCTGTTTGG -3´;  
G, 5´-CGGGGTACCCTGCGCAACGCTACGAAGAAAATGATCTACCAAGCTAA-3´;  
H, 5´-CCGCTCGAGTCATCCTCCATCGCTGCTGCTCTCTGTC -3´;  
I, 5´-ATAAGAATGCGGCCGCTATGAGCTTTGATC -3´;  
J, 5´-
Western blot analysis

Equal amounts of protein were separated by SDS-PAGE, and the proteins were transferred onto nitrocellulose membrane by electroblotting. The blots were probed with a 1:4,000 dilution of goat anti-PTB polyclonal antibody (Santa Cruz Biotechnology), 1:4,000 dilution of 4F4 mouse anti-hnRNP C1/C2 monoclonal antibody (Immuquest), 1:6,000 dilution of rabbit anti-Unr polyclonal antibody (a kind gift of Dr. Jackson), 1:1,000 dilution of rabbit anti-cyclin B1 polyclonal antibody, or 1:4,000 dilution of mouse anti-β actin monoclonal antibody (ICN Biomedicals). Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody against mouse (Amersham Pharmacia Biotech), rabbit (Amersham Pharmacia Biotech) or goat (Santa Cruz Biotechnology) immunoglobulin. For detection of overexpressed Flag–tagged hnRNP C1, blots were incubated with 1:1,000 horseradish peroxidase-conjugated mouse anti-Flag M2 monoclonal antibody (Sigma). Proteins were revealed with an enhanced chemiluminescence kit (NEN Renaissance, Perkin-Elmer).

Transient transfection and reporter gene assay

DNA was transiently transfected in human embryonic kidney HEK293T cells by calcium phosphate precipitation (O'Mahoney and Adams, 1994). For the RNA interference assays, HEK293T cells were seeded at a density of 1.5x10⁵ cells per well in a 12-well plate. The following day they were transfected with 20 pmoles of siRNA duplex (Dharmacon: si none: D-h001210-01-05; si hnRNP C1/C2: M-011869-00; si unr: M-015834-00 ; si Fluc: D-001206-13-05) and 100 ng reporter plasmid using Dharmafect 2 transfection reagent (Dharmacon) according to the manufacturer’s instructions. Six hours later the transfectants were split in wells of a 12-well plate. Lysates were prepared 48 hours afterwards using 1x Passive Lysis Buffer (Promega). Renilla and firefly luciferase activities were measured using
the dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions, and light emission was detected by a Topcount scintillation counter (Perkin-Elmer).

**Cell Culture, cell cycle arrest and cell cycle synchronization**

Human embryonic kidney HEK293T cells (a gift from Dr. M. Hall) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin, 1% streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. In order to arrest HEK293T cells in the prophase of mitosis, cells were treated with 1 µM nocodazole for 16 hours. HEK293T cells were synchronized by treatment with 5 mM thymidine for 24 hours to arrest the cells in the G1/S transition. To release this block, the cells were washed 3 times with PBS and grown in medium without the 5 mM thymidine excess. The IL-3-dependent mouse pre-B cell line Ba/F3 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 10% (v/v) conditioned medium from the WEHI-3B cells as a source for IL-3.

**Time-lapse imaging microscopy**

Thirty-two hours after transfection, a cover glass on which transfected cells were grown was assembled into a LUDIN chamber (Life Imaging Services, Switzerland). Time-lapse microscopy of cells expressing hnRNP C1-GFP and PTB-HcRed was carried out using a Leica AS MDW live cell imaging system (Leica Microsystems, Mannheim, Germany). This system includes a DM IRE2 microscope equipped with an HCX PL APO 63x/1.3 glycerin-corrected 37°C objective and a 12 bit CoolSnap HQ Camera. The microscope is equipped with an incubation chamber, which keeps the cells at 37°C in a 5% CO₂ environment. Differential interference contrast and fluorescence images were taken every 2.5 minutes for 16 hours long. The GFP and HcRed fluorescence images were monitored using a monochromator system with a 75Watt Xenon lamp for excitation in combination with a standard B/G/R filtercube. Resolution of the images was enhanced using the Huygens deconvolution software (Scientific Volume Imaging BV, Hilversum, The Netherlands). In the process of 3D rendering (lower row in figure 5C) cells that interfered the visibility of the diving cell were removed.
**Cell cycle analysis**

DNA content was assayed by flow cytometric analysis of propidium iodide (PI) uptake. Twenty-four hours after siRNA transfection, the cells were detached and seeded into petri dishes. Forty-eight hours later the cells were collected by trypsin treatment, and subsequently washed twice in PBS. Prior to freezing, the cells were resuspended in PBS containing 0.1% sodium citrate and 75 µM PI. Flow cytometric analysis of PI uptake was performed with a Becton Dickinson FACScalibur.

Mitotic index was determined by analysis of chromosomal condensation. Twenty-four hours after siRNA transfection, the cells were detached and seeded onto cover glasses. Forty-eight hours later the cells were washed with PBS and fixed in 4% paraformaldehyde. Then they were washed three times with PBS and either mounted in DAPI containing Vectashield mounting medium (Vector) or permeabilised in PBS by 0.3% Triton X-100 (Roche Diagnostics Belgium N.V.). Permeabilised cells were incubated for 30 min in PBS containing 0.3% BSA and 0.025 mg/ml RNAse A (Roche Diagnostics Belgium N.V.). Finally, the cells were washed once with PBS and mounted in PI containing Vectashield mounting medium (Vector). DAPI stained nuclei were examined with a Zeiss Axiophot fluorescence microscope in order to determine the percentage of cells in mitosis. Analysis of the mitotic index of PI stained cells was performed by identifying condensed nuclei by laser scanning cytometric analysis (Compucyte, Cambridge) of cellular DNA content and maximal PI pixel density. The percentage of mitotic cells was calculated using Olympus LSC Compucyte software.
Supplementary data_Fig1: Cell cycle analysis of a synchronized cell population.

Figure 1: Ba/F3 were synchronized in G1 by IL-3 depletion for 14 hr. Cell extracts and propidium iodide stained samples were prepared at indicated time points after restimulation with IL-3. Western blot analysis was performed using anti-cyclin B1 and anti-actin antibodies. The mitotic index was determined visually by fluorescence microscopy. Σ indicates an asynchronous cell culture.
Figure 2. HnRNP C1 stimulates UNR IRES mediated translation from an intact bicistronic mRNA. Di-c-myc, Di-pRF-UNR and Di-pFR-UNR in combination with an empty or hnRNP C1 expression vector and a β-galactosidase expression vector were transfected in HEK293T cells. Forty-eight hours after transfection the cells were lysed and luciferase and galactosidase activities were measured. The bars represent the average (n=2) ± S.D. luciferase activities, normalized by the corresponding galactosidase activities. Normalized luciferase activities of si none transfected cells were set as 100%.
Supplementary data_Fig3: Unr inhibits UNR IRES mediated translation form an intact bicistronic mRNA.

Figure 3. Unr inhibits UNR IRES-mediated translation form an intact bicistronic mRNA. Di-pRF-UNR or Di-pFR-UNR in combination with an empty vector or hnRNP C1 expression vector and a β-galactosidase expression vector were transfected in HEK293T cells. Forty-eight hours after transfection the cells were lysed and luciferase and galactosidase activities were measured. The bars represent the average (n=2) ± S.D. of the luciferase activities, normalized by the corresponding galactosidase activities. Normalized luciferase activities of si none transfected cells were set as 100%.