Inhibition of translational initiation by let-7 microRNA in human cells
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Supporting Online Materials

Materials and Methods

Construction of plasmids

The RL and FL constructs bearing let-7 sites and BoxB sequences. To obtain Renilla luciferase (RL) expression from a CMV promoter, the RL coding region was released from phRLTK (Promega) as a NheI-BamHI fragment and inserted into similar sites in the pClneo vector (Promega) to get pRL-Con. Note that this RL gene contains optimized codon usage (humanized) for better expression in mammalian cells. To make RL reporters whose expression is controlled by endogenous let-7a RNA, we inserted annealed primers into the XbaI-NotI sites of pRL-Con to get constructs having one perfectly base-pairing (ACTATACAACCTACTACCTCA; pRL-Perf), one bulged (GCACAGCCTATTGAACCTACCTCA; pRL-1xBulge), three bulged (GGACAGCCTATTGAACCTACCTCACTCGGAGCACA GCCTATTGAACCTACCTCAAGCTGCACAGCCTATTGAACCTACCTCA; pRL-3xBulge) and three mutated bulged (GCACAGCCTATTGAACCTACCTCAC TCGAGCACAGCCTATTGAACCTACCTCAAGCTGCACAGCCTATTGAACCTACCTCA CGCTC: pRL-3xBulgeMut) let-7 sites in the 3’UTR. The mutated bulged binding sites have a 2-nt insertion (underlined) in the seeding region of the bulged let-7 complementary site. We verified that this mutation abolishes the repressive effect of endogenous let-7 RNA on translation of the RL reporter (figs. S9B and C). To obtain firefly luciferase (FL) constructs with let-7 complementary sequences, we initially modified the pGL3 Promoter plasmid (Promega) by insertion of a T7 RNA polymerase promoter into the HindIII site upstream of the FL ORF. The resulting plasmid was named pFL-Con. To obtain pFL-Perf and pFL-3xBulge, containing one perfect and three bulged let-7 sites, respectively, the corresponding XbaI-HpalI fragments were excised from the 3’UTR regions of pRL-Perf and pRL-3xBulge and inserted to corresponding sites of
A derivative of pRL-Con, pRL-5BoxB, containing 5 BoxB hairpins in the 3’UTR, was described before (S1).

To generate control plasmids used in ER-targeting experiments, plasmids pRL-Con, pRL-5BoxB and pRL-3xBulge were modified by inserting appropriate annealed oligonucleotides into the *Nhe*I site, upstream of the RL ORF. These plasmids (HA-RL series) allow for expression of RL with an N-terminal HA tag. To achieve ER-targeting, sequences encoding a fusion of the calreticulin signal peptide (MLLSVPLLGLGLAVADRSHTGGMAYPYDVPYA; the signal peptide sequence is underlined) with the HA tag were inserted into the N-terminal region, and sequences encoding an ER retention signal KDEL were inserted into the C-terminal region of the protein (ER-HA-RL plasmid series).

**IRES constructs.** To obtain pEMCV-FL-Con, a *BamHI-NcoI* fragment containing the T7 promoter and the EMCV IRES was excised from the plasmid pCREL (S2) and inserted into the *HindIII-NcoI* sites, upstream of the FL ORF in pFL-Con. Similarly, the same *BamHI-NcoI* fragment was inserted into the *BglII-NcoI* sites upstream of the RL ORF in phRL-TK (Promega) to yield pEMCV-RL-Con. To obtain pEMCV-FL-3xBulge and pEMCV-RL-3xBulge, the 3’UTR fragment containing the 3xBulge region was released from pRL-3xBulge as an *XbaI-BamHI* fragment and inserted into similar sites of pEMCV-FL-Con and pEMCV-RL-Con, respectively. To generate pHCV-FL-Con, we PCR-amplified the region encompassing the HCV IRES and the downstream FL ORF from the plasmid pT7HCV-Luc (S3) and cloned it downstream of the T7 promoter in the pCRII-TOPO vector (Invitrogen). A construct pHCV-FL-3xBulge was obtained by a strategy similar to the one outlined above for the EMCV IRES constructs. Further details are available on request.

**Plasmids for tethering of eIF-4E and eIF-4G.** Plasmids pFL-2BoxB-RL and pFL-6BoxB-RL, in both the control and 3xBulge versions, were obtained as follows. First, plasmids pFL-2BoxB and pFL-6BoxB were prepared by insertion of two or six BoxB hairpins into the *XbaI* site in the 3’UTR of pFL-Con. The inserted sequences eliminate the 5’ end *XbaI* site. Subsequently, the *NheI-XbaI* fragments containing the coding and 3’UTR regions of
pFL-2BoxB and pFL-6BoxB were cloned into the *NheI* site, upstream of the RL ORF in phRL-TK (Promega). In the resulting plasmids, the distance between the termination codon of the upstream FL cistron and BoxB hairpins is 95 nt, and that between BoxB hairpins and the downstream RL cistron ATG is 75 nt. Finally, the let-7 sites sequences were inserted into the RL 3′UTR as described above.

The mutant version of the human eIF4GI (amino acids 612-1560, Accession no. AF104913) used in this study lacks the eIF4E-interaction potential but retains capacity to drive productive translation when tethered to a reporter mRNA (S4). For expressing the NHA-fusion of the eIF4GI mutant, the required coding region was PCR amplified from the plasmid pcDNA3-HA-eIF4GI (S5) and inserted into *EcoRI-NotI* sites of the pCIneo λN-HA vector (S1). The mutant form of mouse eIF4E that lacks the cap-binding activity (in this mutant the three-amino-acid sequence PMW at positions 100-102 is replaced by valine; Accession no. NM_007917), is similar in phenotype to a W102L mutation described previously (S6). The eIF4E mutant was kindly provided to us by Dr. N. Sonenberg of McGill University, Montreal. Correctness of all plasmids was verified by sequencing. Additional details of cloning and plasmid sequences are available on request.

*Other plasmids*: Constructs expressing NHA-fusions of LacZ, hAgo2, hAgo4 and the ΔPRP deletion mutant of hAgo2, and the HA-tagged version of hAgo2 were described earlier (S1). A construct expressing the NHA-tagged hAgo3 (Accession no. AK022827) was made by inserting the coding region of the protein into the pCIneo NHA vector (S1). Plasmid expressing Dep1a-GFP was described before (S7).

**In vitro transcription and analysis of RNA**

All plasmids were linearized with *HpaI* and transcribed in vitro by the T7 RNA polymerase, using either the Message Machine kit or the Megascript transcription kit (Ambion) to generate m7G-capped or non-capped RNAs, respectively. Subsequently, RNAs were treated with DNase I and polyadenylated using the *Escherichia coli* poly(A) polymerase (Ambion) according to manufacturer’s protocol. Subsequently, the RNA was purified using the Absolutely RNA Miniprep kit (Stratagene). To verify quality and size of the RNAs, they were electrophoresed on a 1% agarose gel under denaturing
conditions, and either visualized under UV or subjected to Northern analysis using $^{32}$P-labelled random-primed probes specific for RL or FL coding regions. The length of the poly(A) tail was estimated as approximately 250 residues, as based on a comparison with the RNA Century Marker (Ambion), run in parallel. RNA concentrations measured by the UV absorption at 260 nm were occasionally readjusted taking into account the PhosphorImager (Molecular Dynamics) quantification of Northern blots (the probes used for hybridization were complementary to the coding region, which is identical in different RNA forms). Activity of all in vitro synthesized RNAs was determined by translation in the wheat germ extract or the rabbit reticulocyte lysate (both from Promega) according to manufacturer’s instructions. Luciferase activities were measured using the Dual-Luciferase Assay kit (Promega). RNA transfection experiments were reproduced with the two independent preparations of in-vitro-synthesized RNAs.

**Transfections**

HeLa, HEK293, Huh7 and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with L-glutamine (2 mM) and 10% heat-inactivated fetal calf serum (FCS), and maintained using standard protocols. HeLa or HEK293 cells were transfected using Lipofectamine Plus as suggested by the manufacturer. All plasmids used for transfection were prepared using the Maxiprep kit (Qiagen). Unless indicated otherwise, HeLa cells were grown in a monolayer in 6-well plates and 100 ng per well of the reporter plasmid was co-transfected with 50 ng of either pFL-Con or pRL-Con, serving as normalization controls. When RNA was subjected to Northern analysis, an additional 75 ng of a GFP-expressing plasmid was co-transfected to serve as an mRNA level normalization control. When indicated, 2’-O-Me antisense oligonucleotides complementary to either let-7a RNA (UCUUCACUAUACCAACCUCUCAACCUCUU) or the liver-specific miRNA miR-122a (UCUUCACAAACACCAUUGUCACACUCCAACCUU; both from Microsynth) were co-transfected at a final concentration of 100 nM. The let-7Mut and wild-type let-7 siRNA-like duplexes had following sequences: let-7Mut (sense UGAGGGGUUGGUUGUGUCUG, antisense GACAACCUCACCACCCCCUUAAUU) and let-7 wild-type (sense UGAGGUAGUAGGUUGAUAGU, antisense
For DNA transfections, the results are represented as means of three transfections, with one of the conditions (as indicated in the figure legends) set to 1, +/- SD. For DNA transfections, the cells were lysed for luciferase activity measurements 48 h post-transfection. When required, the proteasome inhibitors MG132 and Z-Leu-Leu-Leu-al (both from Sigma) were added to the cell culture medium 4-6 h prior to lysate preparation at final concentrations of 50 μM.

For RNA transfections, the indicated amounts of RNA were transfected into HeLa cells using Lipofectamine Plus or Lipofectamine 2000. Cells were harvested 6 h post-transfection and luciferase activities were measured. All transfection experiments, with both DNA and RNA, were always done in triplicates and repeated at least 3 times.

**Stably transfected cells and cell lines**

HeLa cells were grown in a monolayer in 10 cm dishes and transfected with plasmids pRL-3xBulge, pRL-3xBulgeMut, pClneo HA-Ago2, pClneo NHA-Ago2 or pClneo NHA-Ago3, using Lipofectamine as recommended by the manufacturer. Two days post-transfection, cells were split into a 15 cm plate and selection carried out in the presence of 500 μg/ml of G418 for 16 days. Single colonies were isolated for stably transformed cells expressing HA-Ago fusions and expression of proteins was confirmed by Western analysis with anti-HA antibodies. For cells expressing RL reporters, pools of stably-transformed cells were prepared and analyzed for RL protein and mRNA levels by using luciferase activity assay and RT-PCR, respectively. All stable clones and pools were maintained in media containing 300 μg/ml of G418.

**Polysome analysis**

For the hAgo2 tethering experiments, HeLa cells were grown in 10 cm plates and cotransfected, using Lipofectamine, with 1 μg of pRL-5BoxB and 3 μg of the plasmid encoding Ago fusion proteins. To analyze the let-7-mediated repression, HeLa cells were transfected with 1 μg of RL reporters per 10 cm plate. Antisense 2’-O-Me oligonucleotides (final concentration 100 nM) were included in transfections when indicated. 24 h post-transfection, the cells were split into a 15 cm plate and incubated for another 24 h to maintain them in active growing conditions. Aliquotes of cells were
always removed and grown in a well of a 6-well plate; these cells were used for activity measurements to confirm the degree of repression, prior to lysate preparation. When indicated, the cells were pre-treated with either 30 mM NaF (S8) or 10 μM of homoharringtonine (Sigma) (S9) for 25 min before lysate preparation. The lysate preparation and sucrose gradient centrifugation were as described previously (S8). Briefly, cells were treated with 100 μg/ml of cycloheximide for 5 min, followed by washes with phosphate-buffered saline (PBS) and a hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1.5 mM KCl, 2.5 mM MgCl2, 100 μg/ml cycloheximide). Lysates were prepared by scraping the cells in lysis buffer [hypotonic buffer containing 0.5% deoxycholate, 0.5% Triton X-100 and 120 U/ml of Rnasin (Promega)]. In an alternative procedure, cells were collected by scraping in PBS, transferred to Eppendorf tubes for additional washes and lysed in the lysis buffer containing 200 μg/ml of yeast tRNA. Lysates were centrifuged for 8 min at 3,000 x g at 4°C and supernatants were then layered onto 10-50% sucrose gradients and spun in an SW40 rotor (Beckman) at 36,000 rpm for 2 h at 4°C. Twelve fractions were collected, treated with Proteinase K, and RNA extracted with phenol/chloroform and precipitated with ethanol. RNA samples were treated with DNase I, extracted again with phenol/chloroform and precipitated. Northern analysis was as described earlier (S1). 32P-labelled probes were prepared using the random priming kit (Roche) and fragments of RL, FL, and mouse β-actin cDNAs as templates

**Sub-cellular fractionations**

For preparation of ER-enriched fractions, HeLa cells were transfected with 3 μg of either pHA-RL-5BoxB or pER-HA-RL-5BoxB per 10 cm plate and further split into 15 cm plates as described above. ER membrane fractions were prepared essentially as described (S10). Briefly, cells were harvested by scraping in PBS and disrupted by douncing in a hypotonic buffer. After centrifugation at 1,000 x g for 5 min, the collected supernatants were layered on a 1 ml cushion of 1.3 M sucrose and centrifuged at 14,000 x g in a SW60 Beckman rotor for 2 h at 4°C. The top cytosolic phase and the white interphase (microsomal fraction) above the sucrose cushion were collected. Aliquots of the microsomal fraction were digested with trypsin in the presence or absence of 0.5% Triton X-100. Proteins were resolved by SDS-PAGE. Antibodies for detecting the HA-tagged
fusion proteins and the ER-resident protein ERp57 were from BAbCo and Stressgen Bioreagents, respectively.

For preparation of soluble and pellet fractionations, HeLa cell lines expressing either RL reporters or HA-hAgo2, and Huh7 and control HeLa cells were harvested by trypsinization. When indicated, 2'-O-Me antisense oligonucleotides complementary to either let-7a RNA or miR-122a were transfected into the cells 2 days prior to harvesting at a final concentration of 100 nM. Cells were washed with cold PBS and resuspended in Buffer C [250 mM sucrose, 10 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 2 mM DTT, 30 U/ml RNasin, and one tablet of EDTA-free protease inhibitor cocktail (Roche) per 10 ml] containing 50 mg/ml digitonin (Merck). After incubation on ice for 15 min, samples were centrifuged at 1,000 x g for 5 min at 4°C. The supernatant was re-centrifuged at 14,000 x g at 4°C for 5 min and saved as a cytosolic fraction. The pellet from 1,000 x g centrifugation was washed with Buffer C without digitonin and saved as a cell debris pellet. RNA was isolated from each fraction with Trizol reagent (Invitrogen) and was used for RT-PCR or Northern analysis. When required, proteins present in each fraction were resolved by SDS-10% PAGE and analyzed by Western blotting.

**RT-PCR and miRNA Northerns**

RNA isolated either from intact cells or from sub-cellular fractions was treated with RQ1 DNase at 37°C for 15 min and subsequently with Proteinase K (50 μg/ml) for 15 min at 37°C. It was then phenol extracted and precipitated with ethanol. For reverse transcription (RT), 100 ng of total RNA from each sample was incubated with gene specific primers and 50U of Superscript II RT (Invitrogen). One tenth of the RT reaction was used for PCR. After 15 rounds of PCR, products were analyzed by 6% PAGE, followed by staining with ethidium bromide. The gene-specific RT primers were: AGCCATCATGTTCTTGGCATC (for β-tubulin), CCGCTCTAGAATTACGTTC (for RL), TTACATCACCACACATGGC (for N-Ras), and CTTCTTGCTAAGTCTGAGCC (for K-Ras). Sequences of PCR primers are available upon request.

To detect miRNAs, 10 μg of total RNA prepared from cells or cell fractions was resolved by denaturing PAGE and electroblotted using the semi-dry blotter (Bio-Rad).
Oligodeoxynucleotides complementary to miR-122a and let-7a were 5’-end-labeled, using T4 polynucleotide kinase and [γ-32P]ATP, and used as probes for Northern analysis.

**Immunoprecipitations and Western analysis**

Cells were extracted with Buffer C (see above) containing 150 mM KCl and 0.5% Triton X-100 on ice for 15 min and supernatants were obtained by centrifugation at 14,000 x g for 15 min at 4°C. Supernatants were pre-cleared by incubation with a mixture of pre-blocked Protein A- and Protein G-Sepharose beads for 60 min (pre-blocking was done by incubation for 1 h with 100 μg/ml of BSA). The amount of added beads was always one-tenth of the lysate volume. Supernatants recovered after centrifugation at 2,000 x g were used for immunoprecipitation (IP) reactions. Antibodies were added to the solution at 1:25 dilution and samples were incubated overnight on ice. Pre-blocked Protein A- and Protein G-Sepharose beads were washed with Buffer C and added to the lysate incubated with the antibody. After 2 h at 4°C, the beads were centrifuged, washed three times with Buffer C and analyzed by SDS-10% PAGE. Following antibodies were used for IPs and Western analysis: rabbit anti-Xrn1 and anti-Dcp1a (both kindly provided by J. Lykke-Andersen), anti-GFP mAb (Roche) and anti-HA mAb 3F10 (Roche). A control antibody used for IP experiments was anti-GERp78 (Santa Cruz). For Western analysis all antibodies except anti-Xrn1 were used at 1:1,000 dilutions; anti-Xrn1 was used at 1:500 dilution. Rabbit anti-hAgo2 (a kind gift of T. Hobman, University of Alberta, Edmonton), raised against a recombinant hAgo2 protein fragment, was used at 1:1,000 dilution. Note that this antibody cross-reacts with the other three human Ago proteins.

**Immunofluorescence**

For immunofluorescence (IF) analysis, HeLa cell lines were grown in 6-well plates. When required, HeLa or HEK293 cells were transfected with the plasmids expressing HA-tagged Ago fusion protein, using Lipofectamine Plus. 24 h post-transfection, cells were transferred into 8-well-chambers (Falcon) and grown for another 24 h. Cells were fixed in 10% formalin for 15 min at room temperature, washed with PBS and permeabilized and “blocked” for 30 min with a blocking buffer (PBS with 5% normal...
goat serum), containing 0.2% Triton X-100. The cells were then incubated for 2 h with primary antibody diluted in a blocking buffer, washed (3 x 5 min), and incubated with the secondary antibody for 30 min. The cells were again washed with PBS (3 x 5 min), stained with DAPI and mounted in antifade (Mowiol; Hoechst). Samples were visualized using a 100x objective of an Olympus IX70 fluorescence microscope and digitized images were taken with a photonics CH350/L CCD camera (Photometrics; Deltavision SGI System). Wide-field images were deconvoluted and processed using the Bitplane/Imaris programme. The antibodies used were: rat anti-HA mAb 3F10 (1:100 dilution), rabbit anti-hDcp1a and anti-Xrn1 (each at 1:200), rabbit affinity-purified anti-hLsm1 and anti-hLsm4 (S11) (both at 1:400; kindly provided by R. Lührmann, Max-Planck Institute of Biophysical Chemistry, Göttingen). Secondary antibodies were: chicken Alexa-Fluor 488 anti-mouse IgG, goat Alexa-Fluor 488 anti-rabbit IgG, and goat Alexa 594 anti-rat IgG (Molecular Probes); all used at 1:500 dilution.

**In situ hybridizations and microinjections**

HeLa cells were transfected, by the calcium-phosphate co-precipitation procedure, with 1 μg of Dcp1a-GFP, 2 μg of the RL reporter plasmid, and 7 μg of a carrier plasmid. 16 h post-transfection, cells were split onto glass coverslips, and fixed 24 h later. Antisense 2’-O-Me oligonucleotides (final concentration 100 nM) were included in some transfections as indicated.

In situ hybridizations for RL mRNAs were done with a mixture of fluorescent complementary oligonucleotides as described previously (S12), except that formamide concentration was raised to 50% during hybridization and washes. The oligonucleotides had the following sequences:

- RL1: 5’ aT*AgtcagcagctT*catttgttcagT*gagccaccactgaT*a;
- RL2: 5’ aT*tagctggaggcagcgT*taccatgcagaaaaaT*cacggcgttT*a;
- RL3: 5’ aT*cacaaagatgatT*ttctttggaaggtT*cagcagctcgaaccaagT*a;
- RL4: 5’ aT*aatagcgttggaaaagaaT*ccaggctcgaaccaagT*a.

Residues marked as T* correspond to the amino-modified thymidines that were conjugated to Cy3.
In situ hybridizations for let-7 and miR-122a RNAs were done with either locked nucleic acid (LNA; Exiqon) or RNA probes complementary to let-7a and miR-122a RNAs, respectively. RNA probes were synthesized by the in vitro T7 RNA polymerase transcription, using appropriate linearized DNA templates and a mixture of UTP and digoxigenin-UTP (DIG-RNA-labeling kit; Roche). The probes were purified on Quickspin columns (Roche). The resulting miR-122a probes had following sequences: GGGAGCUCUUUCAAAACACCAUUGUCACACUCCAUUAAG (miR-122a probe), GGGAGCUCUUUCAAUGUGGAUUGUCACACUCCUUUAAG (mutant miR-122a probe), and GGGAGCUCUUUAGAUAUUUAGUGUGAUAUAUGCGUUUGGAUGGUUUGGACUUUAAG (pre-miR-122a probe; the underlined regions are complementary to the mature miR-122a or a precursor-specific region, and mutated bases in the miR-122a mutant probe are shown in bold).

LNA oligonucleotides were labelled with digoxigenin, using terminal transferase 3’-DIG-tailing kit (Roche), and were purified on a Quickspin column. The let-7 LNA probes had following sequences: AACUAUACACCUACCUACCACUA (for let-7a), AACAUAAUAAACCAAUCAUCCUCA (for let-7a mutant; mutations are in bold), and UAUCUCCAGUGGUGGUGUGA (for pre-let-7a, the sequence is complementary to pre-let-7 RNA nucleotide positions 34-55).

LNA and RNA probes were hybridized as described above, except that formamide concentration was set to 30% during hybridization and washes. Digoxigenin revelation was done as described previously (S13).

Cy3-labelled pre-let-7 RNA fused to the hammerhead ribozyme was synthesized in vitro as previously described (S14), using a PCR product as template (S15, S16). The fusion RNA undergoes efficient self-processing to yield pre-let-7 RNA containing authentic 5’ and 3’ termini (S15, S16). The RNA was purified on a Quickspin column, diluted in PBS, and injected alone or with wheat germ agglutinin (WGA) to HeLa cell nuclei as described previously (S14). Pictures were collected 1 h post-injection.

Processing of pre-miRNAs to mature miRNAs occurs in the cytoplasm and is catalyzed by Dicer. Neither the endogenous nor ectopically expressed form of this enzyme co-localizes with PBs [(S17); and data not shown], arguing against the possibility...
that the observed localization of the microinjected RNA in PBs represents the pre-
miRNA processing site rather than the site of sequestration of translationally inhibited
mRNPs.

Images were captured with a coolsnap CCD camera (Roper Scientific), on a
DMRA microscope equipped for epi-fluorescence (Leica) with a 100x objective
(Planapo, NA 1.4), and controlled by Metamorph (Universal Imaging). Single plane
images were then deconvoluted with the Huygens software (Bitplane) and mounted with
Photoshop. Images shown in Fig. 3, and figs. S10, S11 and S14 represent fields of 51 x
51 μm. The insets show an enlargement of an indicated region (3 x 3 μm), which is
representative of cellular localization of RL or let-7 RNA and Dcp1a. To facilitate
visualization of the images, the contrast was adjusted differently between the RL-
3xBulge and the RL-3xBulgeMut samples or between RL-3xBulge and RL-Perf samples.
After deconvolution, the foci present in the RL-3xBulge samples were 5-20 times more
intense than the spots seen in the RL-3xBulgeMut or RL-Perf samples. The RL-
3xBulgeMut and RL-Perf spots probably correspond to single mRNA molecules, while
the 3xBulge foci appear to concentrate many mRNA molecules (see also legend to fig.
S11).

Method for estimating the amount of let-7 RNA and RL mRNA in Dcp1 foci.
Deconvolved images were thresholded to remove background signals. For let-7, the
background was estimated from neighboring non-injected cells. Typically, the threshold
values were low, about 5% of the maximal signal intensities. This ensured that most of
the let-7-specific signal could be included in the analysis. Then, the thresholded Dapi
image was used to create a mask, which was applied on the thresholded let-7 image to
remove the signals originating from nuclear RNAs. The total amount of signal in the
resulting let-7 image was then calculated, yielding the amount of cytoplasmic let-7 in this
image. The thresholded Dcp1 image was also used to create a mask, which was applied
on the previous let-7 image to eliminate the let-7 signal not co-localized with Dcp1. The
amount of let-7 signal in the resulting image was then calculated, yielding the amount of
let-7 co-localized with Dcp1. This number was then divided by the amount of
cytoplasmic let-7 to estimate the fraction of cytoplasmic let-7 RNA present in Dcp foci.
To estimate the amount of co-localization occurring by chance, the same analysis was performed with sets of let-7 and Dcp1 images originating from different cells. For each of these unmatched let-7/Dcp1 couples, the cells were first aligned. This was done by first centering their nuclei, and then rotating the cells to align their longest axis.

The procedure for quantifying RL mRNA co-localization with Dcp1a was the same as that for quantifying let-7 RNA in Dcp1 foci. The thresholds used to create the Dcp1 masks were almost identical for RL-3xBulge and RL-3xBulgeMut mRNAs: they averaged to 4.6% and 4.5% of the maximal values in the images, respectively. These low thresholds ensured that RL mRNAs adjacent to Dcp1 foci could be counted as present within the foci.
fig. S1. Pillai et al.
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fig. S2. Pillai et al.
fig. S3. Pillai et al.
fig. S4. Pillai et al.
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fig. S6. Pillai et al.
fig. S7. Pilai et al.
fig. S8. Pillai et al.
fig. S9. Pillai et al.
fig. S10. Pillai et al.

siRNA let-7Mut

siRNA let-7

Dcp1a

RL-3xBulgeMut

Merge
fig. S11. Pillai et al.

Perf

3xBulge

Dcp1a

RL mRNA

Merge
A

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RL-3xBulge
β-Tubulin

RL-3xBulgeMut
β-Tubulin

N-Ras
K-Ras
β-Tubulin

B

Huh7
HeLa

miR122a
let-7a
HA-Ago2

HA-Ago2
HeLa cell line

Xrn1
Dcp1a

fig. S12. Pillai et al.
fig. S13. Pillai et al.

A

Probe (let-7)

Pre-miRNA

Mutant

WT

HeLa

B

Probe (miR-122a)

Pre-miRNA

Mutant

WT

Huh7

HeLa
fig. S14. Pillai et al.
Supplemental Figure Legends

**Fig. S1.** Characterization of RL reporter mRNAs containing let-7 sequences in the 3’UTR. (A) Sequence complementarity of let-7a miRNA to the 3’UTR of RL reporters. (B) Normalized luciferase activity (upper panel) and mRNA levels (northern analyses; middle panels) determined for cells transfected with indicated RL reporters and either pFL-Con or pGFP reference plasmids. The activities are represented as RL/FL ratios with the ratio measured in transfections with pRL-Con set to one. The PhosphorImager quantification of the RL mRNA levels, normalized for the co-expressed GFP mRNA, is shown at the bottom. The values are means of three independent experiments, +/- SD. (C) Control experiment demonstrating that expression of RL-Perf and RL-3xBulge mRNAs in HeLa cells is controlled by the endogenous let-7 RNA. Inhibition of expression of RL mRNAs containing let-7 sites is relieved by co-transfection of the 2’-O-Me oligonucleotide complementary to let-7 but not the liver-specific miR-122a RNA. HeLa cells were transfected with 100 ng of indicated RL reporter plasmids and 50 ng of pFL-Con. The indicated antisense 2’-O-Me oligonucleotides were added at 100 nM concentration. Luciferase activities (expressed as RL/FL ratios, with the ratio measured for pRL-Con assays in the absence of the oligonucleotide set to 1) were measured 48 h post-transfection.

**Fig. S2.** Trypsin-protection assays verify that the ER-HA-RL protein is targeted to the ER lumen. Lysates were prepared from HeLa cells transfected with either pHA-RL-5BoxB or pER-HA-RL-5BoxB, and microsomal membrane fractions were prepared. Aliquots were digested with trypsin either in the presence (solubilized) or absence (intact) of 0.5% Triton X-100, for times indicated (min). Proteins were resolved by SDS-PAGE and detected by Western blotting. Positions of RL (α-HA) and the ER resident protein, ERp57, are indicated. Two left lanes (input) represent aliquots of purified ER fractions used for the analysis. The diffused slower migration of the ER-HA-RL protein may be caused by its glycosylation.
Fig. S3. Polysomal distribution of RL mRNA in extracts prepared from cells transfected with different reporter plasmids. (A) The hAgo2 tethering-associated repression is accompanied by a shift of the RL-5BoxB mRNA towards the top of the gradient. HeLa cells were co-transfected with pRL-5BoxB and plasmids expressing either HA-Ago2 (upper panels) or NHA-Ago2 (middle panels). Cytosolic extracts were then analysed by sedimentation on 10-50% sucrose gradients. The polysomes profiles represent measurements of absorbance at 260 nm (A\textsubscript{260}). RL and endogenous \( \beta \)-actin mRNAs in individual fractions were detected by northern analysis. (Bottom panel) the PhosphoImager quantification of the RL-5BoxB mRNA distribution, expressed as a percentage of total radioactivity present in each fraction. (B and C) The let-7-directed shift of the RL reporter towards the top of the gradient is similar to that induced by initiation inhibitors but occurs without a general change of the polysome profile. HeLa cells were transfected with the indicated RL reporters. When required, either NaF (S8) or homoharringtonine (S9), known inhibitors of translation initiation, were added to cell cultures prior to lysate preparation. Lysates were loaded on 10-50% sucrose gradients and distribution of both RL-3xBulge (B) and RL-Con (C) mRNAs analysed by northern blotting. The A\textsubscript{260} profiles in the presence of harringtonine are not shown but they are similar to those seen in the presence of NaF. The two inhibitors caused a global shift of ribosomes to the top of the gradient, consistent with the initiation block.

Fig. S4. Properties of the in vitro synthesized capped RNAs used for transfection experiments. (A) Translation of the in-vitro-transcribed capped FL reporters, either poly(A)\textsuperscript{+} (200 ng) or poly(A)\textsuperscript{-} (400 ng), in transfected HeLa cells. The values represent means of three transfections, +/- SD. (B and C) Inhibition of expression of capped mRNAs containing let-7 sites is relieved by co-transfection of the 2’-O-Me oligonucleotide complementary to let-7 but not to the liver-specific miR-122a. HeLa cells were transfected with 200 ng of specified polyadenylated RL (B) or FL (C) RNAs and the antisense 2’-O-Me oligonucleotides were included when indicated. Luciferase activities, plotted in arbitrary units, were measured 6 h post-transfection. We have also tested reporters bearing the ApppN cap at the 5’ end. These reporters showed no activity in transfected cells (data not shown). (D-F) In vitro translational activity of RNAs. The
RNAs were prepared by the T7-catalysed in vitro transcription followed by polyadenylation. Identity of extracts used for translation [wheat germ (WG) or reticulocyte lysate (Retic)] and of added RNAs, at specified concentrations, is indicated in each panel. FL and RL activities, in arbitrary units, represent means of three assays, +/- SD. All incubations with extracts were for 30 min and were performed at RNA concentrations that were non-saturating. (G) Integrity of RNAs used for RNA transfection and in vitro translation experiments. The in-vitro-synthesized RNA, either poly(A)$^+$ or poly(A)$^-$ were separated on denaturing agarose gels and visualized by northern blotting. Positions of size markers (in kB) are indicated on the right of panels.

**Fig. S5.** Activity of IRES-containing reporter RNAs. (A) HCV-IRES-mediated translation is immune to repression by let-7 RNA. HeLa cells were transfected with 500 ng of the indicated capped HCV-FL poly(A)$^+$ RNAs. The capped FL RNA was also assayed in parallel. 2’-O-Me oligonucleotides (100 nM) were included as indicated. Luciferase activities measured for transfections containing control (Con) RNAs were set as one. (B) m$^7$G-cap-driven translation of RL reporter (70 ng), but not IRES-driven translation of the co-transfected FL reporter (300 ng) is subject to repression by let-7. Cells were transfected with indicated combinations of two reporter mRNAs and luciferase activities were measured. Normalized activities for both RL and FL are means of three transfections, +/- SD. The EMCV-IRES-containing RNAs used for the experiments shown in Fig. 2B and fig. S5 contained a non-capped 5’ terminus but similar results were obtained when ApppN-capped RNAs were used for transfection (data not shown). (C) m$^7$G-cap-driven translation of FL reporter (70 ng), but not IRES-driven translation of the co-transfected RL reporter (300 ng) is subject to repression by let-7. Cells were transfected with indicated combinations of two reporter mRNAs and luciferase activities were measured. (D) Cap-dependent but not IRES-dependent translation is specifically affected by anti-let-7 2’-O-Me oligonucleotide. Cells were cotransfected with combinations of reporter mRNAs as described in panel (C), along with the indicated 2’-O-Me oligonucleotides. (E-G) In vitro translational activity of IRES-containing RNAs. The RNAs were prepared by the T7-catalysed in vitro transcription followed by polyadenylation. Identity of extracts used for translation [wheat germ (WG)
or reticulocyte lysate (Retic)] and of added RNAs, at specified concentrations, is indicated in each panel. FL and RL activities, in arbitrary units, represent means of three assays, +/- SD. All incubations with extracts were for 30 min and were performed at RNA concentrations that were non-saturating. (H) Integrity of IRES-containing RNAs used for RNA transfection experiments. The in-vitro-synthesized poly(A)$^+$ RNAs were separated on denaturing agarose gels and visualized by northern blotting.

**Fig. S6.** Translation driven by the tethered initiation factors eIF4E and eIF4G is immune to the let-7 repression. (A) Details of this experiment are similar to those of the experiment shown in Fig. 2C, with the exception that the indicated 2’-O-Me oligonucleotides were included in the transfections. (B) Similar experiment as in Fig. 2C except that tethering was mediated by six BoxB hairpins inserted in the intercistronic region.

**Fig. S7.** Transiently expressed human Ago proteins are enriched in PBs in both HeLa and HEK293 cells. Constructs expressing indicated HA-tagged human Ago2-4 proteins (indicated in upper panels) were transfected into HeLa (A) or HEK293 (B) cells. Cells were analysed by immunofluorescence (IF) using anti-HA mAb (top row) and antibodies against indicated PB marker proteins (middle row). Cells were stained with DAPI to visualize the nucleus. Merged images are shown in a bottom row.

**Fig. S8.** Ago proteins localize to PBs in stable HeLa cell lines. Cell lines stably expressing HA-Ago2 (A), NHA-Ago2 (B) and NHA-Ago3 (C) were analysed by IF using anti-HA mAb (top row) and antibodies against indicated PB marker proteins (middle row). Cells were stained with DAPI to visualize the nucleus. Merged images are shown in a bottom row. (D) Interaction of HA-Ago2 with PB components in the stable HA-Ago2 HeLa cell line. The cell lysate was subjected to immunoprecipitations (IP) with antibodies indicated on the top of each panel and immunoprecipitates were probed by Western blotting with the antibodies shown on the right of each panel. (E) NHA-Ago2 but not its ΔPRP mutant interacts with Dcp1a. HeLa cells were co-transfected with a plasmid expressing HA-tagged Ago fusion proteins indicated at the bottom of each panel,
and with a plasmid expressing GFP-Dcp1a. Other details were as described above. Note that the same cell lysates were used for IPs shown in both panels and that ΔPRP mutant migrates in the gel with mobility similar to that of the full-length protein (S1).

**Fig. S9.** Characterization of HeLa cells stably expressing either tagged Ago proteins (A) or RL reporters (B-C). (A) Comparison of expression of HA-tagged Ago2 and Ago3 with expression of endogenous Ago proteins. Lysates from indicated cell lines were resolved by 8% SDS-PAGE and analyzed by Western blotting. An anti-HA mAb was used for detection of tagged Ago proteins and a rabbit polyclonal anti-Ago antibody (this antibody, raised against a recombinant hAgo2 fragment and kindly provided by Dr. T.Hobman, cross-reacts with all human Ago proteins) for detection of endogenous proteins. Note that tagged NHA-Ago2 and NHA-Ago3 migrate in a gel appreciably more slowly than the endogenous (Endog. Ago) proteins, which do not separate from HA-Ago2. The data indicate that tagged Ago proteins are expressed at levels significantly lower than endogenous proteins. (B) RL activities in lysates prepared from pools of HeLa cells stably expressing either RL-3xBulge or RL-3xBulgeMut reporters. A mutation introduced to the let-7 RNA binding sites in RL-3xBulgeMut (insertion of two C residues; indicated by an arrow) is shown at the top. The two cell lines expressed RL reporter mRNAs at comparable levels, as determined by semi-quantitative RT-PCR (bottom panel; see also fig. S11). (C) The repressed nature of the RL-3xBulge mRNA is confirmed by transfection of the 2′-O-Me oligonucleotide complementary to let-7 RNA to RL-3xBulge-expressing cells. This treatment results in approximately 7-fold stimulation of RL activity. Similar treatment of HeLa cells expressing RL-3xBulgeMut RNA has no effect on RL activity.

**Fig. S10.** Co-transfection of the let-7Mut but not let-7 siRNA-like duplex results in accumulation of RL-3xBulgeMut RNA in PBs. HeLa cells were co-transfected with either let-7Mut (upper panels) or wild-type let-7 (lower panels) RNA duplexes (100 nM), and vectors expressing RL-3xBulgeMut (visualized in red) and GFP-Dcp1a (visualized in green; 800 and 200 ng per 35 mm plate, respectively). Cells were then processed for in situ hybridization and fluorescence analysis. Fluorescent images were deconvolved.
Merged images are shown in right panels. The images shown in the upper row are the same as ones shown in a lower row of Fig. 3A.

**Fig. S11.** Localization of RL-Perf and RL-3xBulge mRNAs in transfected HeLa cells. HeLa cells were transfected with indicated RL reporters and analysed as described in Materials and Methods. The RL-3xBulge RNA localizes in foci, which are adjacent to or overlapping with PBs. The RL-Perf RNA does not colocalize with PBs. To facilitate visualization of the images, the contrast was adjusted differently between RL-3xBulge and the RL-Perf samples. After deconvolution, the foci present in 3xBulge samples were 5-20 times more intense than the spots seen in RL-Perf samples. The RL-Perf spots likely correspond to single RNA molecules (S18), while the 3xBulge foci appear to concentrate many RNA molecules. Each image represents a field of 51 x 51 μm. Insets represent enlargements of indicated regions (3 x 3 μM).

**Fig. S12.** The RL-3xBulge reporter, and miRNP and PB components are enriched in pellet fractions prepared from cells permeabilized with digitonine. (A) RT-PCR analysis of RNA extracted from S14 supernatant (Sup.) and pellet fractions of HeLa cells stably expressing indicated RL reporters either with or without transfection of indicated 2’-O-Me oligonucleotides. Total, analysis of RNA extracted from cells not subjected to fractionation. Lanes “–RT” represent PCR reactions with the reverse transcription step omitted. PCR products were resolved by 6 % PAGE and stained with ethidium bromide. Identity of analyzed mRNAs is indicated on the left of each panel. (B) Indicated cultured cells were permeabilized with digitonine and the supernatant and pellet fractions were analyzed for the distribution of miRNP or PB components indicated on the right of individual panels. The top two panels are northern analyses with 10 μg of RNA extracted from cells either before (Total) or after fractionation. The bottom three panels are western analyses of fractions from HeLa cells stably expressing HA-Ago2, using anti-HA, anti-Xrn1, and anti-Dcp1a antibodies.
**Fig. S13.** In situ hybridizations with mutant and pre-miRNA-specific probes confirm specificity of let-7 RNA (A) and miR-122a (B) detection. HeLa and Huh7 cells were hybridized with either LNA (A) or RNA (B) probes labeled with digoxigenin.

**Fig. S14.** Appearance of the cytoplasmic let-7-specific foci, observed in nuclear microinjection experiments, is prevented by the coinjection of wheat germ agglutinin (WGA). HeLa cells were microinjected with Cy3-labelled pre-let-7 RNA, with (+WGA) or without (-WGA) addition of wheat germ agglutinin, and fixed 1h post-injection. The RNA was visualized by fluorescence microscopy, and Dcp1a by IF using anti-Dcp1a antibody. The boxed region shows a zoomed section highlighting colocalization of the RNA with Dcp1a.
**Table S1.** Fraction of cytoplasmic let-7 RNA and RL reporter mRNAs co-localized with Dcp1a in PBs. The fraction of cytoplasmic RNA present in PBs was measured as described in the Materials and Methods. The numbers represent means (± SD) for at least 10 cells from experiments similar to those described Fig. 3 and fig. S11. "Random" represents the amount of co-localization calculated to occur by chance.

<table>
<thead>
<tr>
<th></th>
<th>Fraction of cytoplasmic RNA present in PBs (%, ± SD)</th>
<th>Range (%)</th>
</tr>
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<tbody>
<tr>
<td>RL-3xMut</td>
<td>3.5 ± 0.7</td>
<td>2.3 - 4.2</td>
</tr>
<tr>
<td>RL-3xBulge</td>
<td>20.7 ± 9.7</td>
<td>10.7 - 36.6</td>
</tr>
<tr>
<td>RL-Perf</td>
<td>2.6 ± 0.6</td>
<td>1.5 - 5.2</td>
</tr>
<tr>
<td>Let-7</td>
<td>21.8 ± 8.1</td>
<td>13 - 40</td>
</tr>
<tr>
<td>Random</td>
<td>1.3 ± 1.1</td>
<td>0.2 - 4.5</td>
</tr>
</tbody>
</table>
### Table S2. Blockage of let-7 by the 2'-O-Me oligonucleotide prevents accumulation of RL-3xBulge mRNA in PBs. HeLa cells were co-transfected with 2'-O-Me oligonucleotide complementary to either let-7 or miR-122a (final concentration 100 nM), and vectors expressing GFP-Dcp1a and RL-3xBulge RNA (200 and 800 ng per 35 mm plate, respectively). Cells were then processed for in situ hybridization with probes specific for RL mRNA, and the fraction of RL RNA positive cells displaying RL RNA accumulation in Dcp1 foci was counted.

<table>
<thead>
<tr>
<th>2'-O-Me oligonucleotide</th>
<th>Fraction of RL RNA positive cells displaying RNA accumulation in PBs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-let-7</td>
<td>21.2 (n=80)</td>
</tr>
<tr>
<td>Anti-miR-122a</td>
<td>75.7 (n=111)</td>
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Table S3. Amount of let-7 and RL-3xBulge mRNA foci adjacent to or perfectly overlapping with PBs. The data are derived from experiments similar to those shown in Fig. 3 and fig. S10, and are based on analysis of 14 and 10 cells for let-7 and RL-3xBulge RNAs, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Number of co-localizing foci</th>
<th>Fraction perfectly overlapping (%)</th>
<th>Fraction adjacent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7 (14 cells)</td>
<td>96</td>
<td>63</td>
<td>37</td>
</tr>
<tr>
<td>RL-3xBulge (10 cells)</td>
<td>46</td>
<td>63</td>
<td>37</td>
</tr>
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</table>
REFERENCES

S10. R. S. Lerner et al., RNA 9, 1123 (2003).