

(28), although recent studies suggest that disruption of the PCNA-DNMT1 interaction results in only a small decrease in the efficiency of maintenance methylation (25, 29, 30). Together with the activity of UHRF1, these factors likely contribute to the high fidelity of CG maintenance methylation that contributes to epigenetic inheritance.

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MicroRNA Inhibition of Translation Initiation in Vitro by Targeting the Cap-Binding Complex eIF4F

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MicroRNAs (miRNAs) play an important role in gene regulatory networks in animals. Yet, the mechanistic details of their function in translation inhibition or messenger RNA (mRNA) destabilization remain controversial. To directly examine the earliest events in this process, we have developed an in vitro translation system using mouse Krebs-2 ascites cell-free extract that exhibits an authentic miRNA response. We show here that translation initiation, specifically the 5' cap recognition process, is repressed by endogenous let-7 miRNAs within the first 15 minutes of mRNA exposure to the extract when no destabilization of the transcript is observed. Our results indicate that inhibition of translation initiation is the earliest molecular event effected by miRNAs. Other mechanisms, such as mRNA degradation, may subsequently consolidate mRNA silencing.

MicroRNAs (miRNAs) are short [~21 nucleotides (nt) in length] regulatory RNAs encoded within the genomes of organisms ranging from plants to animals. They are implicated in the regulation of a wide variety of biological processes (1–7). miRNAs

act in association with Argonaute (Ago) proteins as components of the RNA-induced silencing complex (RISC) to repress mRNA expression (8, 9). Studies using in vivo systems reported that miRNAs either inhibit translation or lead to the degradation of the target mRNA or proteolysis of the nascent polypeptide (10–17). An important limitation to the in vivo studies is the fact that the outcome of mRNA silencing has been examined hours or days after the initial mRNA target recognition. Thus, the development of an in vitro system is necessary to understand the biochemistry of miRNA function, especially the early steps after the recruitment of RISC to the mRNA.

We chose the mouse Krebs-2 ascites cell extract as an in vitro translation system because it supports efficient translation and exhibits many endogenous and viral translational control mechanisms (18, 19). We generated two

constructs encoding the *Renilla* luciferase (Rluc) open reading frame fused to a 3'UTR (untranslated region) containing or lacking six target sites for let-7 miRNA (termed RL and RL-6xB, respectively), followed by a poly(A) tail of 98 nucleotides (20) (Fig. 1A). On the basis of quantitative real-time polymerase chain reaction (PCR) analyses, we estimated the total concentration of let-7a and let-7f miRNAs [which are the most abundant let-7 miRNAs in human tissues (21)] in the extract at ~150 pM. In vitro transcribed mRNAs were translated at concentrations varying from 3 pM to 3 nM. At the lowest concentration (3 pM), RL-6xB mRNA translation was only 25% of that of RL mRNA (Fig. 1B). In contrast, a decrease in translation was not observed at an mRNA concentration of 3 nM, consistent with a limiting concentration of let-7 miRNAs in the extract (Fig. 1B and fig. S1, A and B). In addition, the same degree of translation inhibition was observed when Rluc activity was normalized against firefly luciferase (Fluc) activity, expressed from Fluc mRNA used as an internal control (fig. S1, A and B). Moreover, the RL and RL-6xB mRNAs were translated with similar efficiency in a wheat germ extract where let-7 is absent (fig. S1C). These results show that target mRNA repression is sensitive to the relative concentrations of the mRNA and miRNA. The degree of inhibition by let-7 miRNA for a 1-hour reaction varied between 35 and 75% among the different extract preparations.

To address the specificity of the inhibition of RL-6xB mRNA translation by let-7, we supplemented the Krebs-2 ascites extract with an antisense 2'-O-methyl (2'-O-Me) oligoribonucleotide complementary to let-7 miRNA. This resulted in an ~2.5-fold increase in translation of RL-6xB mRNA (an increase from 28 to 71% relative to RL mRNA at 10 nM), but had no effect on translation of the control RL mRNA (Fig. 1C). Control 2'-O-Me oligonucleotides tar-

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getting either miR-122a or miR-1 miRNAs failed to relieve translational inhibition even at a concentration of 10 nM (Fig. 1C).

With few exceptions, perfect complementarity of base pairing between the mRNA and the seed region of mRNA is required for re-

pression. Translation of a reporter mRNA containing mutations in nucleotides complementary to the let-7 seed sequences (RL-6xBMut) (10) (Fig. 1A) was almost as efficient (~90%) as control RL mRNA (Fig. 1D), which indicated the importance of the let-7 seed region for inhibition of mRNA expression. These data show that the Krebs-2 ascites cell-free translation system faithfully recapitulates the properties of miRNAs established in vivo.

Analysis of the kinetics of let-7-mediated inhibition revealed a decrease (~25%) in translation of RL-6xB mRNA relative to RL mRNA, first detected after 15 min of incubation (Fig. 2A); a stronger inhibition occurred at later times. The 15-min delay may reflect the time required to assemble the RISC complex on the mRNA. Consistent with this idea, preincubation of the reaction mixture at 16°C for 20 min (conditions that are unfavorable for translation) resulted in 30 to 50% inhibition as early as 10 min after transfer to 30°C (Fig. 2B and fig. S2), which demonstrated that the let-7 miRNA-associated machinery, once assembled on the mRNA, rapidly represses translation.

To determine whether the differential inhibition of RL-6xB mRNA expression might be the result of its faster degradation relative to control mRNA, we examined mRNA decay by measuring the integrity of uniformly labeled mRNAs. Both RL-6xB and RL decayed with similar kinetics during translation for up to 40 min of incubation, which indicated that degradation could not account for the differences in translation at the early time points (Fig. 2C). A 13% (±2) difference in stability between RL and RL-6xB mRNAs was observed at 1 hour (Fig. 2C, top). This difference in mRNA stability was abolished when anti-let-7 2'-O-Me was included in the reaction (Fig. 2C, bottom). These results indicate that mRNA degradation is not responsible for the inhibition of translation mediated by let-7 seen at early times.

A salient advantage of the in vitro translation extract over the published in vivo systems is that the step of translation that miRNAs inhibit can be precisely determined. Thus, we examined 80S ribosomal complex formation by performing glycerol gradient centrifugation. At 10 min of incubation, after preincubation at 16°C for 20 min [resulting in 50% inhibition of translation (Fig. 2B)], formation of the 80S complex on the RL-6xB mRNA was reduced by about half when compared with RL, and this inhibition was almost fully relieved in the presence of anti-let-7 2'-O-Me (Fig. 3). These results demonstrate that let-7 miRNA rapidly inhibits ribosome recruitment to the mRNA.

To determine whether let-7 targets the cap-recognition step in translation initiation, we examined the translation of a reporter mRNA containing the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) (Fig. 4A). Internal translation initiation by the EMCV IRES requires neither a functional cap

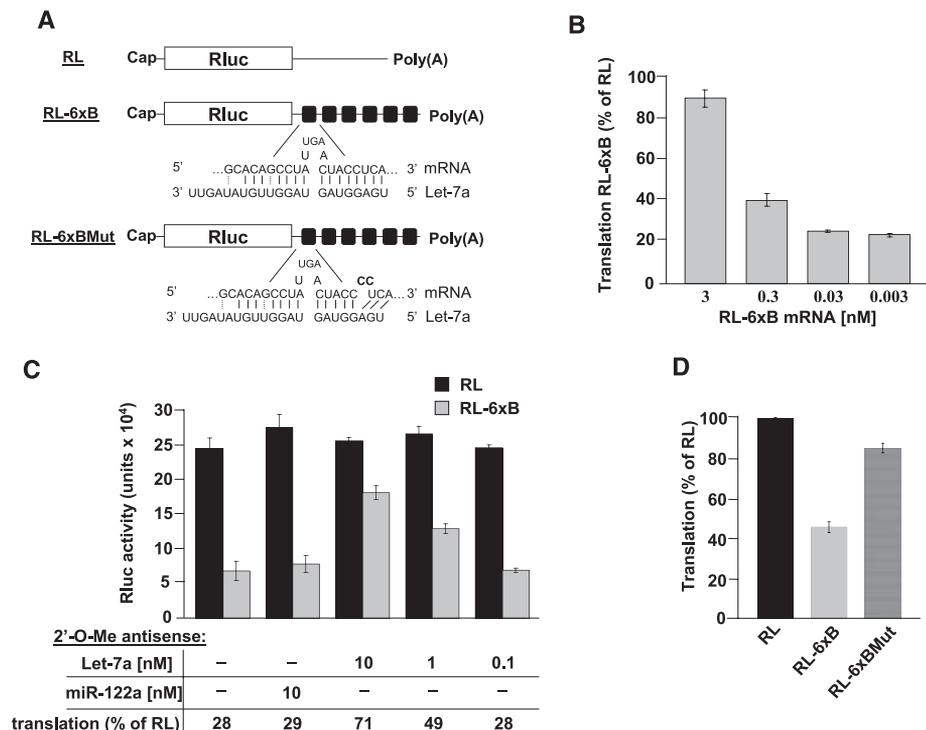


Fig. 1. Repression of protein synthesis by let-7 miRNA in an ascites extract. (A) Schematic representation of the *Renilla luciferase* (Rluc) reporter mRNAs. Sequences of the let-7a-binding sites (RL-6xB) and mutated seed sites (RL-6xBMut) are shown below the drawings. (B) Effect of mRNA concentration on RL-6xB inhibition. (C) Relief of RL-6xB mRNA repression by anti-let-7 2'-O-Me oligonucleotide. Where indicated, antisense 2'-O-Me oligonucleotides to let-7a or miR-122a were incubated for 20 min at 30°C with the extract before a 1-hour translation reaction. (D) Requirement for intact let-7 seed sequence for efficient RL-6xB inhibition. Experiments in (C) and (D) were performed at an mRNA concentration of 0.03 nM. Values represent means ± SD for n ≥ 3.

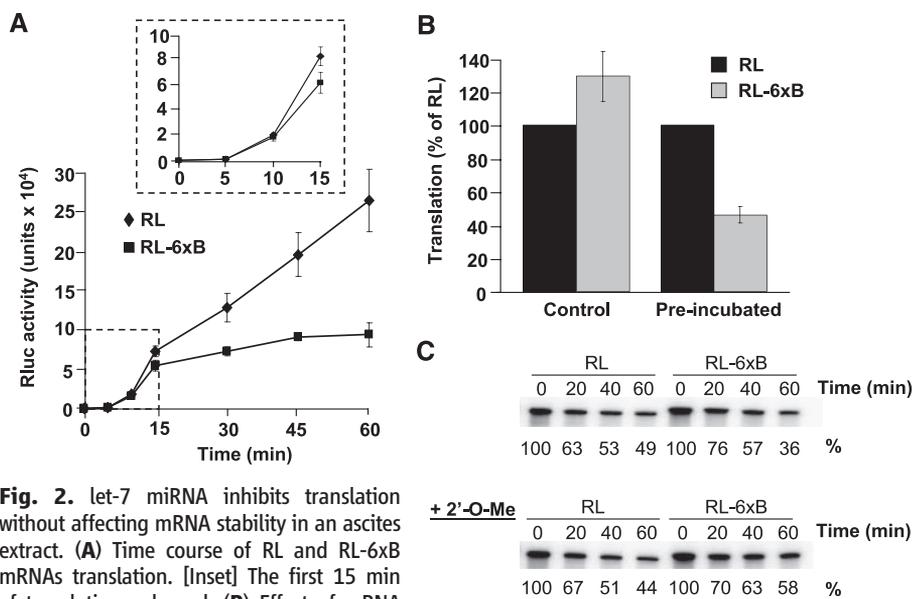


Fig. 2. let-7 miRNA inhibits translation without affecting mRNA stability in an ascites extract. (A) Time course of RL and RL-6xB mRNAs translation. [Inset] The first 15 min of translation enlarged. (B) Effect of mRNA preincubation on RL-6xB repression. Preincubation was carried out at 16°C for 20 min, followed by incubation for 10 min. (C) Time course of mRNA stability, as determined by autoradiography in the absence (top) or presence (bottom) of 10 nM anti-let-7 2'-O-Me oligonucleotide. Percent sign indicates percentage of intact mRNA relative to starting time. The data shown are representative of three independent experiments. Values in panels (A) and (B) represent means ± SD for n = 3.

nor the cap-binding initiation factor eIF4E, a component of the eIF4F complex (22). The EMCV-RL-6xB mRNA was translated effi-

ciently (90%) when compared with the control EMCV-RL mRNA, and the anti-let-7 2'-O-Me oligonucleotide did not stimulate its transla-

tion (Fig. 4B). Thus, we conclude that EMCV IRES-dependent translation is refractory to inhibition by let-7. Next, we substituted the 7-methylguanosine cap (m⁷GpppG) with ApppG on the reporter mRNAs. Although the translation of the ApppG-capped mRNA was, as expected, dramatically reduced (1% of m⁷GpppG-capped mRNA), there was no apparent difference in translation between the ApppG-capped RL and RL-6xB mRNAs (Fig. 4C). These results demonstrate the necessity of a functional cap structure for miRNA-mediated mRNA repression.

To test whether the miRNA machinery might directly target the cap-binding complex eIF4F, we added increasing amounts of purified eIF4F to the extract. Addition of eIF4F stimulated overall translation up to fourfold, which demonstrated that eIF4F is limiting in the Krebs-2 ascites extract (fig. S3). At 80 ng/μl and higher eIF4F concentrations, translation of RL-6xB mRNA increased to the level of control RL mRNA or RL-6xB mRNA translated in the presence of anti-let-7 2'-O-Me (Fig. 4D and fig. S3). Other initiation factors, such as eIF2, failed to reverse miRNA translational inhibition. These data support the conclusion that the miRNA machinery (miRISC) inhibits translation initiation by targeting the m⁷G-cap-recognition process.

In this report, we demonstrate that extracts derived from mouse Krebs-2 ascites cells faithfully recapitulate critical features of miRNA function in vivo. Our results provide a compelling argument in favor of translation initiation as the primary and first target of miRNAs. Our in vitro data are in agreement with some of the findings made in vivo with let-7 and other miRNAs (10, 11). They are also consistent with recent observations that the Ago2 protein binds directly to the m⁷G-cap and that miRNAs repress translation initiation in *Drosophila* embryo extracts (23, 24). However, in contrast to *Drosophila* extracts, no sequestration of repressed mRNAs to pseudo-polysomes occurred in Krebs-2 ascites extract. Our findings are at apparent variance with other data demonstrating miRNA translational control at the step of the 60S ribosome joining during initiation (25), at postinitiation steps (12, 26), or via protein degradation (15). The differences observed in cell transfection assays could reflect cell-specific mechanisms to consolidate the rapid, but milder, translation initiation inhibition revealed here. A recent study reported that miRNA-mediated translational repression can be reproduced in a commercial rabbit reticulocyte lysate (27). However, the recognition of the target in this system was not directed by the endogenous RISC, but required the preannealing of a synthetic miRNA to the mRNA, making it unclear whether the reticulocyte lysate recapitulates the physiological loading of the miRISC on the mRNA.

The levels of many miRNAs change in cancer (28), whereas eIF4E and eIF4G function as oncogenes (29). Our results raise the intriguing possibility that eIF4E and eIF4G function to

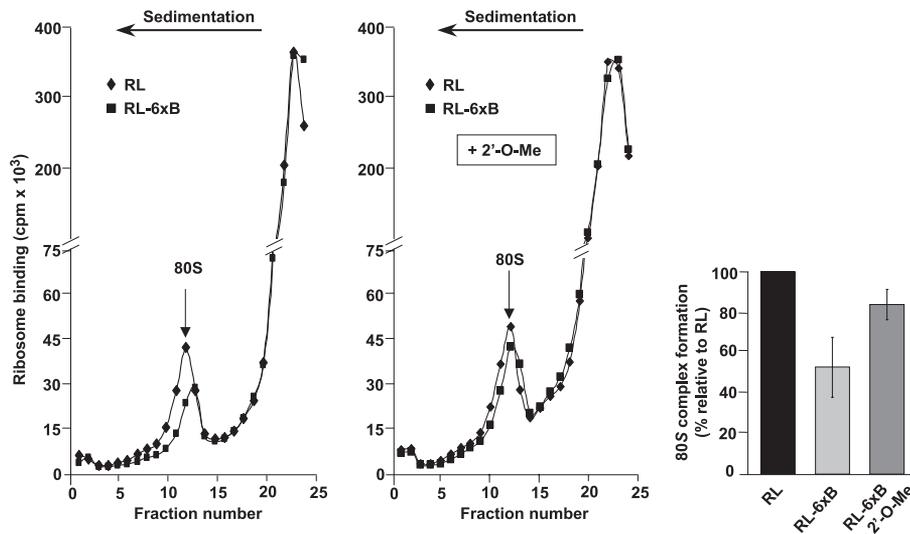


Fig. 3. let-7 miRNA inhibits ribosome binding. Ribosome binding assay of RL and RL-6xB mRNAs in the absence (left) or presence of anti-let-7 2'-O-Me (middle). Ascites extract was preincubated at 16°C for 20 min, followed by a 10-min incubation at 30°C. The position of the 80S ribosomes was determined by recording absorbance at 254 nm. mRNA concentration was 0.03 nM. The right panel represents the percentage of the area under the 80S peak, calculated from three independent experiments, relative to RL. The number of counts per minute in each fraction was normalized to correct for differences in total cpm recovery between RL and RL-6xB gradients. (Right) Values represent means ± SD for *n* = 3.

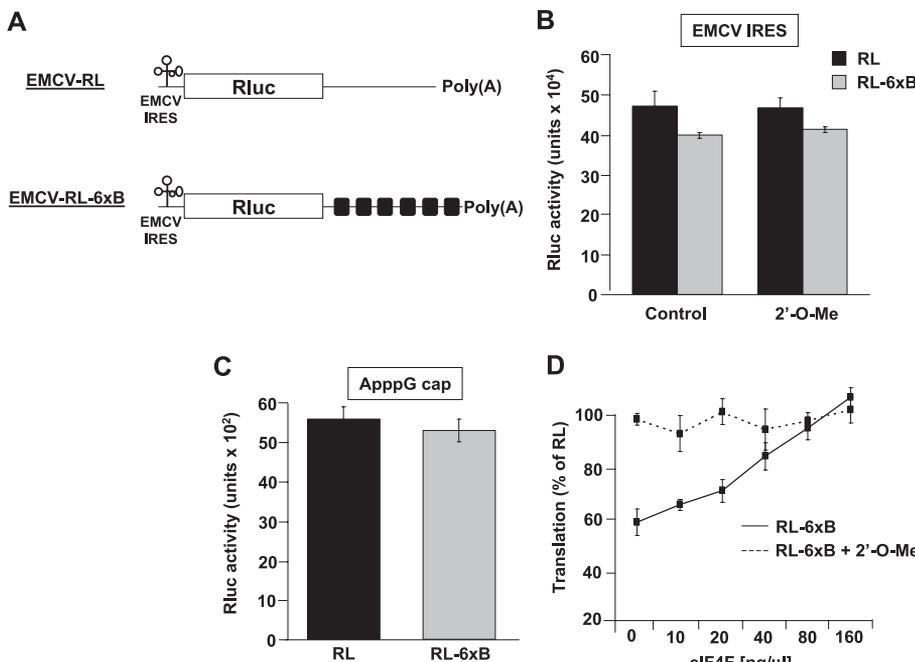


Fig. 4. let-7 miRNA inhibits cap-dependent, but not cap-independent, translation in an ascites extract. (A) Schematic structure of mRNAs containing the EMCV IRES. (B) The EMCV IRES confers resistance to repression of RL-6xB translation. EMCV IRESs containing mRNAs were translated for 1 hour in the presence or absence of anti-let-7 2'-O-Me oligonucleotides. (C) ApppG-capped RL-6xB mRNA is refractory to let-7-mediated inhibition. (D) Effect of eIF4F addition on miRNA-mediated repression. Extracts were supplemented with the indicated amounts of purified eIF4F, and Rluc activity was assayed after a 1-hour translation reaction. Data are depicted as a percentage of translation relative to RL in the presence or absence of anti-let-7 2'-O-Me oligonucleotide. Assays contained 0.03 nM mRNA. Values in (B), (C), and (D) represent means ± SD for *n* = 3.

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counteract the activity of miRNAs in cancer development and progression.

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Materials and Methods

Figs. S1 to S3

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Dynamic Visualization of Thrombopoiesis Within Bone Marrow

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Platelets are generated from megakaryocytes (MKs) in mammalian bone marrow (BM) by mechanisms that remain poorly understood. Here we describe the use of multiphoton intravital microscopy in intact BM to visualize platelet generation in mice. MKs were observed as sessile cells that extended dynamic proplatelet-like protrusions into microvessels. These intravascular extensions appeared to be sheared from their transendothelial stems by flowing blood, resulting in the appearance of proplatelets in peripheral blood. In vitro, proplatelet production from differentiating MKs was enhanced by fluid shear. These results confirm the concept of proplatelet formation in vivo and are consistent with the possibility that blood flow-induced hydrodynamic shear stress is a biophysical determinant of thrombopoiesis.

Blood platelets are required to maintain hemostasis in mammals. The relative paucity of MKs in normal BM contrasts with the relative abundance of platelets in peripheral blood and implies that platelet assembly and release are highly efficient, dynamic processes. The prevalent model for thrombopoiesis, the proplatelet or flow model (1, 2), receives support mostly from MK differentiation cultures (3–5), which deprive MKs of cellular contacts and

signals found in intact BM (6, 7). The flow model proposes that MKs extend plump pseudopodia that give rise to long (>100- μ m) branched proplatelet processes that appear “beaded” by virtue of intermediate swellings (3, 4, 8). It is unclear whether proplatelets detach from MKs in bulk and fragment further into platelets (4) or whether barbell-shaped platelet pairs detach exclusively from proplatelet ends (8), but eventually the MK cytoplasm is exhausted as a result of fragment release. Previous in situ imaging approaches, such as electron microscopy, helped define the morphology and environment of MKs in the BM (1, 2, 9), but they provided only static snapshots that leave room for alternative mechanistic concepts of thrombopoiesis. For example, the platelet territory model proposes that vesicles from internal MK demarcation membranes are released as mature platelets (10–12). Another conundrum pertains to how newly formed platelets traverse BM vessel walls and escape activation by subendothelial prothrombotic factors.

To observe thrombopoiesis in vivo, we studied mouse skull BM by multiphoton intravital microscopy (MP-IVM) (13, 14). To identify MKs and their progeny, we used *CD41-EYFP*^{ki/+} mice in which enhanced yellow fluorescent protein (EYFP) was expressed as a targeted transgene from the endogenous gene locus for *CD41*, an MK- and platelet-specific integrin (15). We used heterozygous *CD41-EYFP*^{ki/+} mice and confirmed that EYFP⁺ MKs in BM of these animals generate fully functional platelets at normal frequency (fig. S1).

MKs showed considerable morphological diversity; they occurred mostly as isolated cells but occasionally in clusters, and they were always found in close contact with BM sinusoids (Fig. 1, A and B, and movie S1). MKs were largely sessile, exhibiting minimal migratory tracks or three-dimensional (3D) instantaneous velocities as compared with other BM-resident cell types (Fig. 1C). To facilitate imaging of normally rare MKs, we pretreated some mice with thrombopoietin (TPO), which increased MK numbers but did not alter their perivascular localization (fig. S2 and movie S2). Another physiologic activity of TPO is the differentiation of immature MKs, which are small and compact, toward larger mature cells that assemble and release platelets (16). Indeed, 3D reconstructions of intravital recordings (Fig. 1, D and E) revealed that TPO treatment increased maximal MK diameters (Fig. 1F) and volumes (Fig. 1G) and caused more irregular MK shapes (Fig. 1H).

Many MKs exhibited fragmented protrusions (Fig. 1, I and J, and movie S3), whereas others were surrounded by scattered EYFP⁺ particles (Fig. 1A and movie S4), which may represent proplatelets that remained connected to the MK cell body. However, particle connections were mostly inferred based on near-linear alignment and close proximity of particles to large MK bodies (Fig. 1J), because insuffi-

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MicroRNA Inhibition of Translation Initiation in Vitro by Targeting the Cap-Binding Complex eIF4F

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