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Human let-7a miRNA blocks protein production on actively translating polyribosomes

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MicroRNAs (miRNAs) regulate gene expression at a post-transcriptional level through base-pairing to 3′ untranslated regions (UTRs) of messenger RNAs. The mechanism by which human let-7a miRNA regulates mRNA translation was examined in HeLa cells expressing reporter mRNAs containing the *Caenorhabditis elegans* lin-41 3′ UTR. let-7a miRNA strongly repressed translation, yet the majority of control and lin-41–bearing RNAs sedimented with polyribosomes in sucrose gradients; these polyribosomes, together with let-7a miRNA and the miRISC protein AGO, were released from those structures by puromycin. RNA containing the lin-41 3′ UTR and an iron response element in the 5′ UTR sedimented with polysomes when cells were incubated with iron, but showed ribosome run-off when the iron was chelated. These data indicate that let-7a miRNA inhibits actively translating polyribosomes. Nascent polypeptide coimmunoprecipitation experiments further suggest that let-7a miRNA interferes with the accumulation of growing polypeptides.

miRNAs are evolutionarily conserved noncoding RNAs ~21 nucleotides (nt) in length that regulate gene expression at the post-transcriptional level by base-pairing to partially complementary sequences in 3′ UTRs of target mRNAs[1]. miRNAs control several biological processes in worms, flies, zebrafish and mammals, including developmental timing, cell differentiation, cell proliferation, apoptosis and patterning of the nervous system[2,3]. In addition, the mutation or misexpression of miRNAs correlates with various human cancers, indicating that they might act as tumor suppressors or oncogenes[4].

let-7a miRNA regulates developmental timing in the nematode *C. elegans* and controls the expression of several transcription factors, including the 'RING, B-box, coiled-coil' (RBCC) protein LIN-41, which functions as a translational repressor of the transcription factor LIN-29 during the larval-to-adult transition[5–7]. let-7a miRNA is also present in humans, where it has been implicated in lung-malignancy formation[8,9].

In *C. elegans*, the lin-4 miRNA target mRNAs lin-14 and lin-28 sediment with polyribosomes, although little LIN-14 or LIN-28 protein has been detected, indicating that mRNA expression is inhibited after translation initiation[10,11]. This post-initiation repression is also suggested by the observation that miRNAs sediment with polyribosomes in mammalian cells and in worms[10,12,13]. Together, these results imply that miRNAs stall ribosomes during translation elongation. However, in mammalian cells transfected with short interfering RNAs that can function as miRNAs[14], ribosomes are not stalled, but are released prematurely from the mRNA[15]. In contrast, other studies found that mRNA under miRNA control sediments with nontranslating ribonucleoproteins (RNPs) in sucrose gradients[16]. Moreover, miRNAs cannot repress the translation of a reporter RNA containing an internal ribosome entry site (IRES)[16,17], which indicates that miRNAs regulate cap-dependent initiation.

These experiments suggest that miRNAs might function at multiple levels, which prompted us to examine the mechanism by which the *C. elegans* lin-41 3′ UTR, containing two phylogenetically conserved let-7a miRNA sites[5,8], controls gene expression in transfected HeLa cells. Although the lin-41 3′ UTR strongly repressed mRNA expression, it did not prevent mRNA sedimentation in the polysome-containing portions of sucrose gradients. The lin-41–containing RNA was engaged in translation, as evidenced by its sensitivity to puromycin, which causes premature polypeptide termination and polysome breakdown. The insertion of an iron response element (IRE) into the 5′ UTR of the lin-41–containing RNA had no effect on the polysome sedimentation of a reporter RNA in the presence of iron, but when the iron was chelated and further initiation was consequently inhibited, ribosome run-off took place. Finally, although an RNA lacking the lin-41 3′ UTR was immunoprecipitated from polysomes by way of the nascent polypeptide, an RNA containing the lin-41 3′ UTR was not. Together, these results suggest that one function of let-7a miRNA is to interfere with the accumulation of growing polypeptides on normally translating polyribosomes.

RESULTS

Human let-7a miRNA represses translation in HeLa cells

To investigate the mechanism by which miRNAs repress mRNA translation in human cells, we transfected HeLa cells with plasmids

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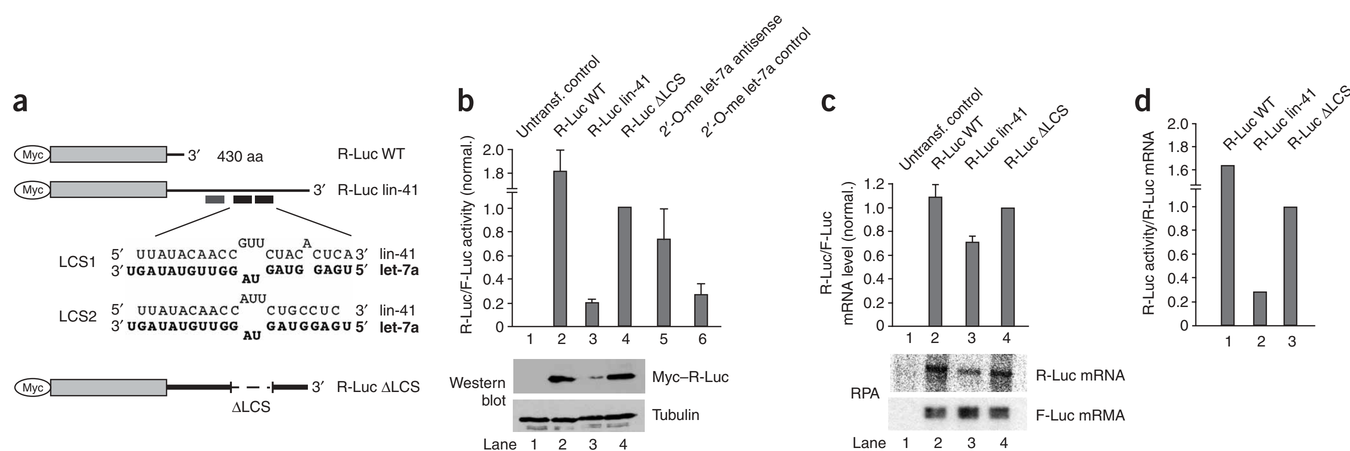

Figure 1 let-7a miRNA–mediated translational repression in HeLa cells. (**a**) Schematic of mRNA reporter constructs used in this study. *Renilla* luciferase containing an eight–Myc epitope tag at the N terminus (R-Luc WT) was appended with either the *C. elegans* lin-41 3′ UTR (R-Luc lin-41) containing two let-7a miRNA target sites (LCS1 and LCS2, black bars)[5,18] and one putative lin-4 miRNA site (dark gray bar), or the lin-41 3′ UTR lacking LCS1 and LCS2 (R-Luc ΔLCS). (**b**) Endogenous let-7a miRNA efficiently represses lin-41 UTR–containing reporter RNA translation. HeLa cells were transfected with plasmids encoding RNAs in **a**, or firefly luciferase as an internal control, and the normalized *Renilla*/firefly luciferase activity ratio was measured. Cells were also cotransfected with a 2′-O-methyl (2′-O-me) RNA oligonucleotide complementary to let-7a miRNA or, as a control, an oligonucleotide with a scrambled sequence. Bars represent mean values ± s.d. from four independent experiments, normalized to value obtained with R-Luc ΔLCS (arbitrarily set to 100%). Expression of Myc-tagged *Renilla* luciferase was visualized in independent experiments by immunoblotting; tubulin served as a loading control. (**c**) RNase protection assay (RPA) of steady-state mRNA abundances. RNA was extracted from HeLa cells transfected with indicated plasmids and used for RPA analysis. R-Luc WT and R-Luc lin-41 RNAs were compared to R-Luc ΔLCS RNA, whose protection was set to 100%. As in **b**, *Renilla* RNA values were normalized to the firefly RNA values, which were determined by RPA in parallel. Bars represent mean values ± s.d. from three independent experiments. (**d**) Normalized values of *Renilla* luciferase activity (**b**) were divided by normalized *Renilla* mRNA abundances (**c**) to estimate net effect of let-7a miRNA–mediated mRNA turnover on protein expression.

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encoding N-terminally Myc epitope–tagged *Renilla* luciferase appended with the *C. elegans* lin-41 3′ UTR, which contains two experimentally verified let-7a miRNA target sites separated by a 27-nt intervening spacer sequence[5,18] (**Fig. 1a**). Control plasmids encoded identical reporter RNAs that lacked the lin-41 3′ UTR (R-Luc WT) or that contained the lin-41 3′ UTR but without the let-7a sites (R-Luc ΔLCS) (**Fig. 1a**). When quantified by dual luciferase assays, protein abundance was shown to be reduced about ten-fold in HeLa cells transfected with R-Luc lin-41, compared with cells expressing R-Luc WT (chart in **Fig. 1b**, bars 2 and 3). Deletion of the let-7a sites in the lin-41 3′ UTR efficiently restored expression of the reporter by about five-fold (**Fig. 1b**, bar 4). Moreover, luciferase activity increased by about four-fold when a 2′-O-methyl–containing RNA oligonucleotide complementary to let-7a miRNA was cotransfected with R-Luc lin-41 RNA, but not when a control oligonucleotide was used (**Fig. 1b**, bars 5 and 6). The expression of Myc-tagged *Renilla* luciferase reporter proteins was visualized independently by western blotting analysis (blots in **Fig. 1b**).

In similar transfection experiments, R-Luc lin-41 RNA abundance was ∼1.4-fold lower than R-Luc ΔLCS RNA abundance (**Fig. 1c**), indicating that let-7a miRNA induces some mRNA turnover, as reported previously[19,20]. These results were independently confirmed by quantitative real-time (RT)-PCR analysis (data not shown). When *Renilla* luciferase activity was normalized to the corresponding mRNA abundances, let-7a miRNA was shown to reduce the corresponding protein abundances by ∼3.5-fold (**Fig. 1d**), demonstrating that the lin-41 3′ UTR represses mRNA expression via let-7a miRNA in human cells. This observation is consistent with the finding that miRNAs repress gene expression by a dual mechanism involving both translational repression and post-transcriptional reduction of mRNA abundance[20,21].

Repressed mRNAs associate with translating ribosomes

We next analyzed the distributions of the reporter mRNAs on polysome sucrose gradients. Cytoplasmic extracts prepared from HeLa cells transfected 24 h earlier were centrifuged through 15%–50% gradients; after fractionation, the sedimentation profiles of the RNAs were determined by RNase protection assays (**Fig. 2a**). R-Luc WT and R-Luc lin-41 RNAs were nearly indistinguishable in their distributions in the gradients: ∼60%–65% of each RNA sedimented into the polyribosome-containing fractions (fractions 4–10). A similar sedimentation profile was obtained with R-Luc ΔLCS RNA (data not shown). We also examined the pellet obtained by the initial centrifugation step at 10,000*g* (see Methods) and found that there was no differential loss of reporter RNAs (data not shown). About 85% of tubulin RNA, which served as an internal control, sedimented into the polysome fractions. To assess whether the RNAs in the polysome regions were translated, the HeLa cells were treated with puromycin, a drug that causes premature polypeptide termination and polysome disassembly[22]. Puromycin (400 μM) not only caused the dissociation of polysomes, as determined by UV absorbance, but also induced a shift in the sedimentation of both reporter RNAs as well as endogenous tubulin RNA to the lighter, nontranslating, RNP-containing part of the gradients (fractions 1–3) (**Fig. 2a**). These results indicate that both reporter RNAs were actively translated in HeLa cells. In addition to let-7a, the lin-41 3′ UTR contains a target site for the *C. elegans* lin-4 miRNA[5]; a putative human homolog of this RNA is miR125 (ref. 23). Although we did not investigate the abundance of miR125 in HeLa cells, it could conceivably influence the translation or stability of our R-Luc lin-41 reporter RNA. With this caveat in mind, for subsequent experiments we chose to compare R-Luc lin-41 RNA with R-Luc WT RNA, which we assume is devoid of any regulatory information.

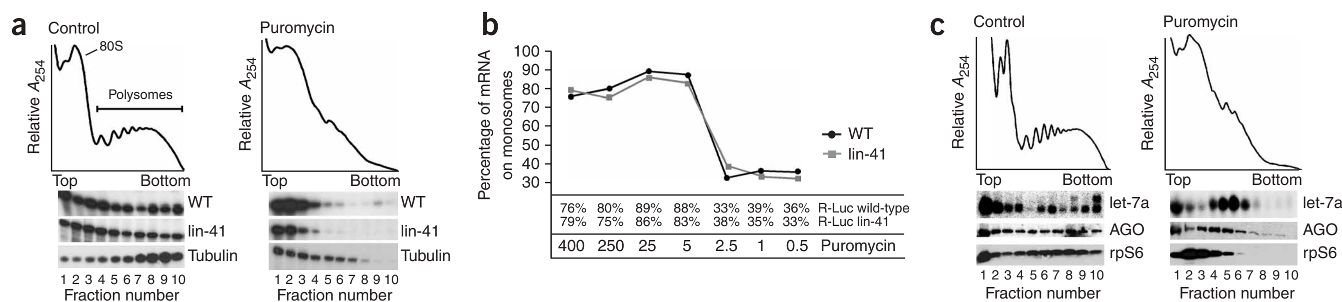

Figure 2 lin-41–containing mRNA and let-7a miRNA are associated with translating polyribosomes. (**a**) HeLa cells transfected with plasmid R-Luc WT or R-Luc lin-41 were subjected to polysome analysis; distributions of reporter mRNAs and tubulin mRNA were analyzed by RPA. Left, control experiments show distributions of R-Luc WT, R-Luc lin-41 and tubulin mRNAs at steady-state abundances. Right, transfected cells were incubated with puromycin before being harvested for polysome analysis in identical experiments. (**b**) Cells were treated with indicated concentrations of puromycin before polysome analysis, and RNase protection assay (RPA) signals were quantified by phosphorimaging to evaluate percentages of total mRNA cosedimenting with monosomes and polysomes. (**c**) HeLa cells incubated in the absence or presence of puromycin (200 μM) were fractionated on sucrose gradients as in **a**, and distribution of let-7a miRNA was assessed by RPA. Fractions 1–4, RNPs; fractions 5–10, polysomes. In similar experiments, distributions of endogenous AGO protein and ribosomal protein S6 were visualized by western blotting.

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To assess whether the polypeptide elongation rates of lin-41–lacking and lin-41–containing RNAs were substantially different, HeLa cells were incubated with a broad range of puromycin concentrations (0.5–400 μM); RNAs with rapidly transiting ribosomes should be particularly sensitive to low amounts of the drug and sediment to lighter portions of gradients. At concentrations as low as 5 μM, puromycin caused both reporter RNAs to sediment mostly in the RNP factions of the gradients; 0.5, 1 and 2.5 μM, however, had little effect on translation of either reporter RNA (**Fig. 2b** and data not shown). These data suggest that RNA under the regulation of let-7a miRNA supports polypeptide elongation at the same rate as unregulated RNA.

Endogenous miRNP complexes associate with ribosomes

We also investigated whether let-7a miRNA as well as members of the Argonaute (AGO) protein family, known as miRISC core components[3], cosediment with polyribosomes, as indicated in studies using other mammalian cell types[12,13]. HeLa cells, some of which were treated with 200 μM puromycin, were fractionated on sucrose gradients, and then let-7a miRNA was analyzed by RNase protection and AGO protein(s) by western blotting (**Fig. 2c**). When untreated cells were fractionated, both let-7a miRNA and AGO were detected in RNP fractions (fractions 1–4) as well as in polysomal fractions (fractions 5–10). By contrast, puromycin treatment caused the polysomal let-7a miRNA and the AGO protein(s) to shift to RNP portions of the gradient. As expected, ribosomal protein S6 was also shifted upon puromycin treatment. Therefore, we conclude that the let-7a–miRISC complex in HeLa cells is at least partially associated with actively translating polyribosomes. We note that, because of their high degree of sequence homology[24] and the similarity of their molecular weights, we were unable to distinguish which of the human Argonaute proteins were detected on our immunoblots.

A block of initiation results in ribosome run-off

To confirm and extend the observation that the mRNA under the control of let-7a miRNA is indeed translated, the sedimentation profiles of reporter RNAs were examined after blocking initiation. For this purpose, we took advantage of a translational-switch system composed of the iron response element stem-loop structure and its cognate RNA-binding protein, iron regulatory protein-1 (IRP-1)[25]. Under conditions of iron deprivation, IRP-1 binds the IRE in the mRNA 5′ UTR and inhibits cap-dependent initiation by preventing 40S ribosomal subunit association with the eIF4F (eIF4E–eIF4G–eIF4A) initiation complex[26] (**Fig. 3a**). Accordingly, HeLa cells were transfected with R-Luc WT or R-Luc lin-41 reporter mRNAs containing the consensus IRE at position +30 in their 5′ UTRs. When available intracellular iron was chelated with deferoxamine mesylate salt (DFMO, 100 μM)[27], luciferase activity and protein abundance derived from IRE_R-Luc WT RNA were reduced to nearly undetectable levels (**Fig. 3b**, bar 3). Thus, IRP-1 effectively inhibited translation of the WT reporter RNA. As expected, R-Luc lin-41 expression was nearly undetectable in the absence or presence of DFMO (**Fig. 3b**, bars 2 and 4). These data demonstrate that the IRE–IRP-1 binary complex acts as a translational switch in human cells. In similar transfection experiments, R-Luc mRNA abundance was analyzed by quantitative RT-PCR (**Fig. 3c**), which showed that R-Luc lin-41 mRNA abundance was ~1.3- to 1.5-fold lower than that of R-Luc WT in the presence of hemin and DFMO. The reduced RNA abundances are thus comparable to those in **Figure 1**. *Renilla* luciferase activity was further normalized to the respective RNA abundances (**Fig. 3d**), revealing that let-7a miRNA caused at least a five-fold reduction in protein accumulation in the presence of the iron response element.

We next analyzed the distribution in polysome gradients of IRE-containing *Renilla* luciferase mRNAs from cells grown in the presence (with hemin) and absence (with DFMO) of iron. The presence of iron resulted in most of the IRE_R-Luc WT (69%) and IRE_R-Luc lin-41 mRNA (61%) cosedimenting with polysomes (**Fig. 3e**, lanes 5–10). After cells were cultured in the presence of DFMO for 12 h, both RNAs sedimented exclusively with nontranslating RNPs at the tops of the gradients (**Fig. 3f**). Notably, neither the polysome profiles nor the sedimentation of tubulin mRNA was affected by hemin or DMFO treatment (**Figs. 3e,f**). The observed sedimentation profiles of the reporter RNAs are typical of ribosome run-off, suggesting that the let-7a miRNA–repressed mRNA is associated with actively translating ribosomes.

It was possible, and indeed likely, that during the 12-h incubation of HeLa cells with DFMO, at least some reporter RNA degradation took place, and that the RNA at the tops of the gradients was composed of both run-off and newly synthesized transcripts. Indeed, the incubation of HeLa cells with actinomycin D for 12 h followed by RNase protection assays revealed that ~60% of the IRE_R-Luc lin-41

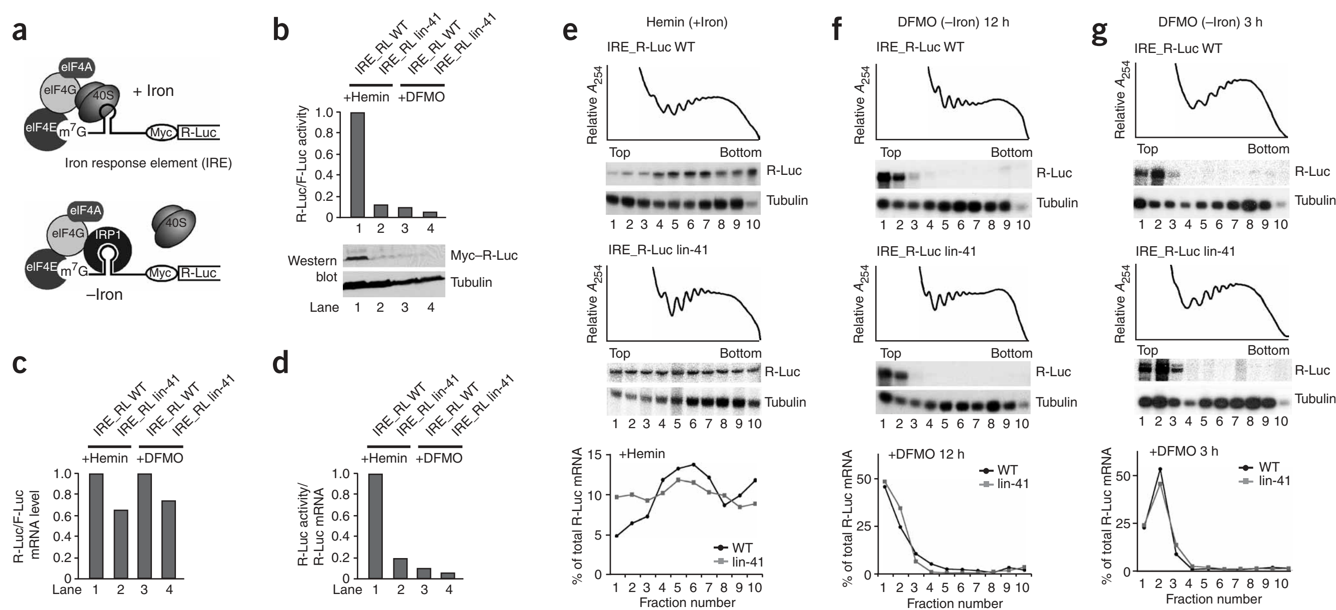

Figure 3 Block of translation initiation results in ribosome run-off from repressed mRNAs. (**a**) Schematic of the translation initiation complex assembled on mRNAs harboring the stem-loop IRE. In the presence of iron, the eIF4F complex (eIF4A–eIF4E–eIF4G) properly recruits the 43S preinitiation complex to the mRNA so that initiation may proceed. In the absence of iron, IRP-1 binds the IRE and prevents eIF4F recruitment of the eIF3/40S subunit, thus blocking initiation[26]. (**b**) IRE-containing reporter RNAs are not translated in HeLa cells cultured in the presence of an iron source. HeLa cells transfected with plasmid IRE_R-Luc WT (bars 1 and 3) or plasmid IRE_R-Luc lin-41 (2 and 4) were cultured with hemin, an iron source (1 and 2), or DFMO, an iron chelating agent (3 and 4). Normalized *Renilla*/firefly luciferase activity ratio was measured 24 h after transfection (top) and *Renilla* luciferase protein abundances were independently visualized by immunoblotting (bottom). Tubulin served as a loading control for blots. Luciferase activities represent averages from three experiments. (**c**) Quantification of steady-state abundances of IRE-containing reporter RNAs in the presence of hemin or DFMO, by quantitative RT-PCR. Relative amounts of IRE_R-Luc RNA in lanes 2–4 were compared with abundance of IRE_R-Luc WT RNA in lane 1 (set to 1.0) and normalized to firefly luciferase RNA abundance, which was determined in parallel. Bars represent mean values from two independent experiments. (**d**) Normalized values of *Renilla* luciferase activity (**b**) were divided by normalized *Renilla* luciferase mRNA abundances (**c**) to estimate net effect on protein accumulation. (**e**) Repressed IRE-containing mRNA (expressed in the presence of iron) cosediments with polyribosomes in sucrose gradients. HeLa cells transfected with plasmid IRE_R-Luc WT or IRE_R-Luc lin-41 were incubated with hemin and subjected to polysome analysis. Luciferase and tubulin mRNA abundances were analyzed by RPA and quantified by phosphorimaging (bottom chart). (**f**,**g**) Sequestration of iron results in ribosome run-off. HeLa cells transfected as in **c** were incubated with DFMO for 12 h (**f**) or 3 h (**g**) before analysis by polysome sucrose-gradient centrifugation and RPA. Bottom charts show quantification of RPA signals by phosphorimaging.

reporter mRNA remained stable during that time (data not shown). Therefore, we incubated HeLa cells with DFMO for only 3 h, during which ~90% of the RNA was stable (data not shown). An analysis of the polysome profiles revealed that, as with the 12-h DFMO treatment, almost no RNA sedimented with polysomes and the vast majority was found in the RNP fraction (**Fig. 3g**). These data further demonstrate that ribosome run-off occurs on both control and lin-41–bearing RNAs.

Nascent polypeptide chain–mRNA coimmunoprecipitation

The results presented thus far seem paradoxical: if mRNA under the control of let-7a miRNA is translated normally, why is almost no protein produced? Perhaps the miRNA signals the destruction of the growing polypeptide chain, or 'tags' the polypeptide so that it is rapidly destroyed after its completed synthesis and release from the polysomes. If so, then the nascent polypeptide might not be recognized by an antibody directed against the N terminus, the first portion that emerges from the 60S ribosomal subunit. To address these possibilities, we transfected HeLa cells with plasmids encoding R-Luc WT and R-Luc lin-41 RNAs that would express luciferase with eight Myc epitope tags fused to the N terminus. A Myc antibody was then used to immunoprecipitate the nascent polypeptide associated with the encoding polysomal mRNAs from a cell extract. The RNA was extracted and examined for the *Renilla* open reading frame by both RT-PCR and RNase protection assays (**Fig. 4a**). Mock immunoprecipitations using nonspecific IgG did not precipitate luciferase RNA to any appreciable extent, irrespective of its 3′ UTR. However, Myc antibody immunoprecipitated the luciferase RNA in the absence, but not the presence, of the lin-41 3′ UTR. These results indicate that the nascent polypeptide chain derived from mRNA under let-7a miRNA control either is destroyed soon after it emerges from the 60S ribosomal subunit or is 'masked' by associated factors that mark the complete protein for destruction soon after it is released from the polysomes (**Fig. 4b**).

DISCUSSION

We have investigated the mechanism of let-7a miRNA–mediated translational repression by transfecting HeLa cells with reporter genes bearing the *C. elegans* lin-41 3′ UTR. Our results strongly imply that this repression occurs at a step after translation initiation, according to the following observations. (i) Repressed mRNAs that cosediment with polyribosomal fractions in sucrose gradients at steady-state level are associated with actively translating ribosomes, as shown by their sensitivity to puromycin. (ii) Components of the endogenous miRNA–RISC complex (let-7a miRNA and Argonaute) also sediment with polysomes and are similarly sensitive to puromycin treatment. (iii) Ribosomes associated with repressed mRNA run off *in vivo* when translation initiation is subsequently

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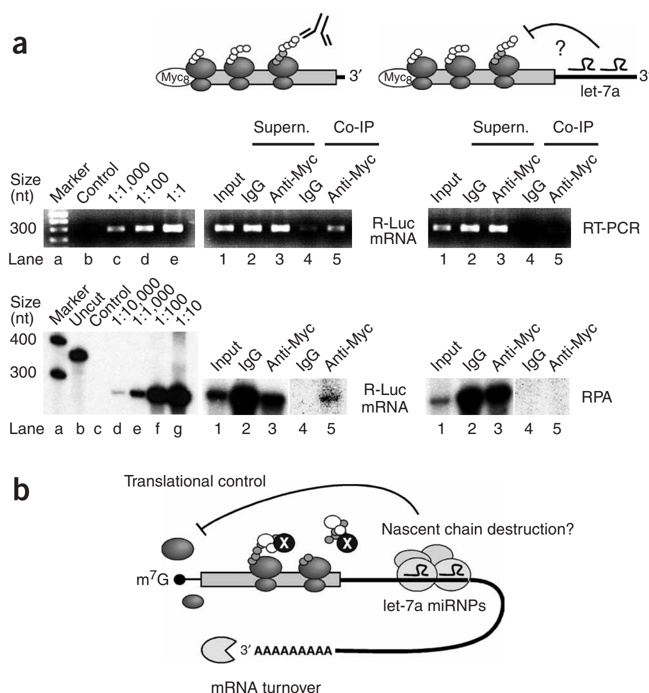

Figure 4 Polyribosomes are not coimmunoprecipitated when under miRNA control. (**a**) HeLa cells were transfected with plasmids encoding lin-41 containing or lacking *Renilla* luciferase reporters tagged with eight N-terminal Myc epitopes. Cell extracts were used for nascent polypeptide and thus polysome immunoprecipitation with anti-Myc (lane 5) or, for a mock control, with nonspecific IgG (lane 4). Using RT-PCR (top gels) and RNase protection (RPA; bottom gels), luciferase mRNA was detected in RNA extracted from the precipitates. Luciferase RNA was also detected from 10% of the input and from 50% (RT-PCR) or 100% (RPA) of unbound (supernatant) material (lanes 1–3). Total RNA isolated form HeLa cells transfected with plasmid R-Luc WT was tested in parallel at the indicated dilutions (left gels), serving as an internal standard. Control lanes contain RNA isolated from untransfected cells. Middle gels show polysome immunoprecipitation of R-Luc WT RNA; right gels show polysome immunoprecipitation of R-Luc lin-41 RNA. (**b**) Model of let-7a miRNA function in human cells. let-7a miRNPs and associated RISC components might interfere with translation initiation, as indicated in ref. 16, and may, in parallel, deposit a specific protease-recruiting factor (X) on the growing polypeptide chain that leads to its destruction. let-7a miRNPs also mediate RNA deadenylation and mRNA turnover, most probably involving human counterparts of the *Drosophila* deadenylase complex CCR4–NOT1 (refs. 19–21).

blocked by endogenous IRP-1 that is tethered to an iron response element within the 5′ UTR. (iv) Ribosomes associated with repressed mRNAs but not with normally translated mRNAs are not coimmunoprecipitated by an antibody directed against an N-terminal Myc epitope of the reporter protein. Together, these data indicate that let-7a miRNA interferes with protein accumulation on normally translating polyribosomes.

Our results contrast with a report of miRNA-dependent ribosome drop-off and premature peptide release when miRNA target sites are located no closer than ~1 kilobase from the termination codon[15]. In the lin-41 3′ UTR that we used, the let-7a sites were ~0.6 kilobases from the termination codon. If premature peptide release had occurred with our lin-41–containing reporter RNA near the normal termination codon, as seen in ref. 15, we most probably would have immunoprecipitated the RNA via the Myc epitopes on the growing polypeptide chain. We also would have detected a difference in the sucrose-gradient sedimentation profiles of this RNA and wild-type RNA at varied puromycin concentrations. We did not observe either of these phenomena.

Our results are also inconsistent with the previous suggestion that miRNAs repress translation at the cap, probably by preventing eIF4E-eIF4G joining[16]. In ref. 16, a sucrose-gradient analysis similar to ours and showed that nearly 50% of the miRNA-regulated RNA sedimented with polysomes. Although the authors did not analyze this material further, we speculate that it could represent translating mRNA whose protein product was rapidly destroyed. We also note that on our gradients, more than 30% of the lin-41–containing RNA sedimented at the top of the gradient, and this material could correspond to RNA blocked at initiation, as described in ref. 16. Thus, miRNAs may regulate mRNA expression at two levels—at initiation and during ongoing translation—to ensure that no functional protein is produced.

Recent experiments have also demonstrated that miRNAs can induce mRNA destruction[19–21]. In our studies, however, we observed only about a 30% reduction in the abundances of reporter mRNAs that are under miRNA control. This value is comparable to those reported in refs. 15 and 16. Moreover, the original studies[10,11] that first described translational control by lin-4 miRNA in *C. elegans* indicated that very little endogenous lin-14 and lin-28 RNA is targeted for destruction. It thus seems that, under a variety of experimental conditions, miRNAs can have largely different effects on RNA turnover. Indeed, miRNA-induced changes in mRNA abundances vary substantially and are not observed for all of the known miRNA targets in *Drosophila melanogaster*[21,28].

Finally, it has been observed that miRNA-repressed reporter mRNA accumulates in ill-defined cytoplasmic foci that are adjacent to P-bodies, cytological structures that may act as storage depots[16]. Although we have not examined the relationship between our lin-41–containing reporter RNA and P-bodies (but see ref. 29), the previous study[16] detected only ~20% of miRNA-repressed mRNA in those structures. This observation implies that at steady state, miRNA-mediated repression does not require an association with P-bodies or P-body–associated foci. Consistent with this view, the dissolution of P-bodies after the reduction of the core component Lsm1 does not abrogate let-7a miRNA–controlled translational repression[30].

Because the proteasome inhibitors MG132 and lactacysteine had no effect on R-Luc lin-41 3′ UTR RNA expression (data not shown), nascent or completed polypeptide destruction is unlikely to be mediated by this proteolytic complex. We therefore speculate that specific proteases or factors that recruit proteases might be associated with the 3′ UTR–tethered miRNA–RISC complex. In regard to this, we note that growing polypeptide chains interact with several ribosome-bound factors, including chaperones[31] and the conserved heterodimeric nascent polypeptide–associated complex (NAC), which is one of the first cytosolic factors that ubiquitously binds emerging polypeptides irrespective of the amino acid sequence[32]. NAC prevents the inappropriate interaction of nascent chains with the signal-recognition particle, thus ensuring that inappropriate targeting of nonsecretory proteins to the endoplasmic reticulum does not occur. Notably, the *Drosophila* gene *bicaudal*, which is important for repression of Nanos mRNA translation—a process that also occurs at a step after initiation—encodes a NAC subunit[33,34]. Similarly, both subunits of *Drosophila* NAC have been implicated in repression of Oskar mRNA translation after initiation[35]. Indeed, more than 50% of nascent polypeptides are cotranslationally degraded in living cells[36], thus underscoring the generality of this process as a mechanism to regulate gene expression.

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METHODS

DNA constructs. Plasmid R-Luc WT was generated by insertion of an eight–Myc epitope tag into the NheI site of vector pRL-TK (Promega). Plasmid R-Luc lin-41 was constructed by insertion of a 1,068-nt fragment of the *C. elegans* lin-41 3′ UTR corresponding to nucleotides 3715–4782 of transcript C12C8.3 in the Ensembl database, using XbaI and NotI restriction sites. Plasmid R-Luc ΔLCS, carrying an 85-nt deletion[5,19] (606–690), was generated by PCR. IRE_R-Luc plasmids were constructed by insertion of a DNA fragment encoding the iron response element[25,27] at position +30 of the *Renilla* luciferase transcript, using the HindIII site of vector pRL-TK. Plasmids used for run-off transcription contained the following cloned DNA fragments (position 1 as defined by the manufacturer): *Renilla* luciferase (pRL-TK, nucleotides 1070–1350), firefly luciferase (pGL3, Promega, 900–1130) and human α-tubulin (pEGFP-Tub, Clontech, 1380–1580).

Cell culture and transfections. HeLa CCL2 cells were maintained in DMEM (Gibco) supplemented with 10% (v/v) FBS (Sigma), glutamine and penicillin-streptomycin according to standard protocols. All transfections were carried out on 90%–95% confluent cells grown in monolayer, using Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h after transfection and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Here, *Renilla* luciferase plasmids were cotransfected in each experiment in triplicate, with the pGL3 vector expressing firefly luciferase at a 5:1 ratio, using 24-well plates. Where indicated, 2′-O-methyl RNA oligonucleotides[37] were cotransfected at a final concentration of 100 nM. Four hours after transfection of IRE_plasmids, media was replaced by fresh DMEM containing either 50 μM hemin (Fluka) or 100 μM deferoxamine mesylate salt (DFMO, Sigma). For experiments shown in **Figure 3f,g**, cells were first grown in media containing 50 μM hemin after transfection and then placed into media containing 100 μM DFMO for 12 or 3 h before they were harvested. For the experiments shown in **Figure 2**, cells were placed in fresh media containing a final concentration of puromycin (Sigma) of 400 μM (1 h treatment) or 250, 25, 5, 2.5, 1 or 0.5 μM (20 min) before harvesting the cells as described below. In other experiments, cells were incubated with actinomycin D at a final concentration of 0.01 μg ml^{-1} for 3 or 12 h.

Polysomes and immunoblotting. HeLa cell extracts used for polysome gradient centrifugation were prepared as described[38]. In brief, HeLa cells cultured in 10-mm culture dishes were harvested 24 h after transfection by replacing the culture media with fresh media containing cycloheximide (Sigma) at a final concentration of 100 μg ml^{-1} for 5–10 min. Cells were washed with PBS, trypsinized, pelleted and resuspended in low-salt buffer (20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$). Triton-X 100 was added to the cell suspension to a final concentration of 0.3% (v/v) and cells were lysed on ice using a 1-ml Dounce homogenizer. The solution was centrifuged for 1 min at 10,000*g* at 4 °C, and supernatants were layered on top of linear 15%–50% (w/v) sucrose gradients (11 ml). Centrifugation was carried out in a Beckmann SW41Ti Rotor at 36,000 r.p.m. for 2 h at 4 °C. Polysome profiles were monitored by absorbance of light with a wavelength of 254 nm (A_{254}). Aliquots of HeLa cell extracts used for polysome analysis were separated in parallel on denaturing 10% SDS-polyacrylamide gels, and the expression of Myc-tagged *Renilla* luciferase was analyzed by immunostaining using the mouse monoclonal antibody 9E10 (Covance, USA) and the ECL detection system (Perkin Elmer). Endogenous tubulin was detected using a mouse monoclonal antibody to α-tubulin (Sigma). For western blotting analysis of sucrose-gradient fractions, proteins were precipitated in the presence of ethanol, separated on denaturing 10% SDS-polyacrylamide gels and immunostained using the mouse monoclonal antibody to AGO, 2A8 (see Acknowledgments), or rabbit polyclonal antibody to ribosomal protein S6 (Cell Signaling).

RNase protection assays. RNase protection assays were carried out according to standard protocols using *in vitro*–transcribed ^{32}P-labeled antisense RNA directed against *Renilla* luciferase, firefly luciferase, human α-tubulin or human let-7a miRNA. *In vitro* transcripts were generated in the presence of 0.8 μM [α-^{32}P]UTP (3,000 Ci $mmol^{-1}$, Perkin Elmer) by T7 run-off transcription. The let-7a antisense probe was designed using the mirVana miRNA probe construction kit (Ambion) and DNA oligonucleotide let-7a (see below). Total RNA from HeLa cells was isolated 24 h after transfection using the RNeasy Kit (Qiagen), including an on-column DNase I treatment. RNA isolation from sucrose-gradient fractions was carried out by proteinase K digestion in the presence of SDS, followed by Trizol (Invitrogen) and phenol/chloroform/isoamyl alcohol (25:24:1) extraction. Hybridization reactions were carried out at 42 °C overnight and RNase digestion was performed at 37 °C for 30–45 min using the RNase A/T1 cocktail (Ambion). Samples were treated with proteinase K, and protected RNA fragments were isolated by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation before they were separated on a 6% (w/v) polyacrylamide, 7 M urea gel and visualized by phosphorimaging.

Nascent-chain immunoprecipitation and quantitative RT-PCR analysis. HeLa cells were grown in 10-mm cell-culture dishes, transfected with plasmid R-Luc WT or R-Luc lin-41 and harvested 24 h after transfection as described above. Monoclonal antibody to Myc (9E10) or nonspecific IgG was coupled to protein A–Sepharose beads, washed with PBS and incubated with 500 μl of freshly prepared HeLa cell extract for 2 h at 4 °C on a head-over-tail mixer. Beads were washed twice with 1 ml of ice cold buffer A (10% (v/v) glycerol, 200 mM sorbitol, 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 12 mM $MgCl_2$). RNA was extracted from the beads as well as from the input and supernatants using the RNeasy Kit (Qiagen), including DNase I treatment, and analyzed by semiquantitative RT-PCR or RNase protection. Total RNA (5 μg) isolated from HeLa cells transfected with plasmid R-Luc WT was used in parallel for RT-PCR analysis at the indicated dilutions, serving as an internal standard. PCR products were separated on a 1% (w/v) agarose gel and visualized by ethidium bromide staining. Quantitative real-time RT-PCR analysis was carried out using the Applied Biosystem ABI Prism 7700 Detection System and Qiagen's SYBR RT-PCR Kit. Total RNA was isolated from HeLa cells 24 h after transfection and treated with DNase I. The relative amounts of *Renilla* luciferase mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method[39] and normalized to the respective abundances of firefly luciferase RNAs.

DNA and RNA oligonucleotides. DNA and RNA oligonucleotides were purchased from IDT, USA, and had the following sequences: let-7a, 5′-TGAGGTAGTAGGTTGTATAGTTTTTTTCCTGTCTC-3′; 2′-O-methyl let-7a antisense, 5′-UCUUCACUAUACAACCUACUACCUCAACCUU-3′; 2′-O-methyl let-7a control, 5′-UCUUCUGAUAUGUUGGAUGAUGGAGUACCUU-3′.

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AUTHOR CONTRIBUTIONS

S.N. performed all the experiments, M.J.S. cloned the lin-41 3′ UTR and S.N. and J.D.R. analyzed the data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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