

Extra View

Cell cycle control of microRNA-mediated translation regulation

Shobha Vasudevan, Yingchun Tong and Joan A. Steitz*

Department of Molecular Biophysics and Biochemistry; Howard Hughes Medical Institute; Yale University School of Medicine; New Haven, Connecticut USA

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MicroRNAs are small regulatory RNA molecules that exert post-transcriptional control overexpression of specific target mRNAs. AU-rich elements (AREs) are highly conserved 3'UTR sequences that alter the stability and translation of mRNAs of clinical importance as a rapid and transient response to external and internal changes. We recently demonstrated that a reporter mRNA containing the tumor necrosis factor α (TNF α) ARE activates translation in response to quiescence via microRNA target sites in the ARE. Further studies revealed that microRNAs in general have the potential to regulate translation in a cell cycle determined manner: in quiescent cells, microRNAs activate translation while in cycling/proliferating cells, microRNAs repress translation.

In this study, we have analyzed microRNA regulation of translation at additional stages of the cell cycle. We observe the strongest repressive potential in the S and S/G₂ phases with minimal repression in the G₁ phase. Since asynchronously growing cells are predominantly in G₁, these data may explain the variability in magnitude of microRNA-mediated repression reported in the literature. Importantly, we observe activation in contact-inhibited G₀ quiescent cells, reaffirming that the quiescent state and not serum-starvation-induced stress causes microRNA-mediated translation upregulation. In addition, we find that siRNPs, unlike microRNPs, downregulate expression of a reporter in serum-starvation-induced G₀ arrested cells, as well as in proliferating cells. Our data underscore the importance of the quiescent state for microRNA-mediated translation activation and suggest the potential for further novel functions of microRNAs in distinct cell fates.

Introduction

We recently demonstrated that luciferase reporters bearing the TNF α ARE or microRNA target sites in their 3'UTRs are regulated at the translation level by the cell cycle.^{1,2} In asynchronous cultures, where the majority of the cells are in the G₁ phase, we observed the translation efficiency (defined in Fig. 1) of the ARE or microRNA target site reporters to be equal to that of the control reporter, which

was therefore called basal translation. When the cells were induced into quiescence (G₀ phase) either by serum starvation or by growth to confluency, the translation efficiency of the test reporter was greater than that of the control reporter, which was therefore called translation activation. When synchronous cultures were in the S/G₂ phase of the cell cycle, we observed that the translation efficiency of the test reporter was less than that of the control reporter, which was therefore called translation repression.² It should be noted that alleviation of translation repression observed under conditions of stress³ causes translation of the test reporter to be restored to the basal level (or to that of the control reporter), and is therefore not the same as translation activation, where the levels are greater than the basal level of the control reporter.

In this study, we have examined more precisely the cell cycle regulation of microRNA-controlled translation and demonstrate translation activation in naturally-achieved quiescent conditions. We provide a detailed protocol for cell cycle synchronization of cultured cells for analysis of microRNA functions. Additionally, our data reveal that while microRNPs can activate translation upon serum-starvation, with siRNPs under identical conditions, only repression is observed.

Results

To monitor the regulation of microRNA-controlled translation at designated stages of the cell cycle, we performed luciferase reporter assays under the following conditions. HeLa cells or HEK293 Tet-on cells, transfected with the test or control Firefly reporters along with the co-transfected Renilla control reporter for normalization, were serum starved to induce cells to exit the cell cycle from G₁ and enter G₀ (often called G₀/G₁ arrest or quiescence; see Discussion). Cells in other distinct cell cycle stages were obtained by first arresting them in either serum-starved G₀ or drug-induced cell cycle phase-specific conditions and then releasing them for synchronized reentry into the cell cycle (Materials and Methods).

We had previously used B-box-containing luciferase reporters to which AGO2 or (as a control) mutant AGO2 (prp Δ) was tethered via fusion to lambda N-protein (see Fig. 1).⁴ We reported that in synchronized proliferating cells 18 hours after release from serum starvation or in nocodazole-treated G₂-arrested cells, the AGO2-tethered reporter demonstrated five-fold translation repression, compared to five-fold translation activation in G₀ cells. In contrast, the control prp Δ -tethered reporter did not show a significant change in translation efficiency. The results with tethered AGO2 were similar to those observed using a reporter containing microRNA

*Correspondence to: Joan A. Steitz; Department of Molecular Biophysics and Biochemistry; Howard Hughes Medical Institute; Yale University School of Medicine; 295 Congress Avenue; BCMM 136E; New Haven, Connecticut 06536 USA; Tel.: 203.737.4418; Fax: 203.624.8213; Email: joan.steitz@yale.edu

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target sites upon addition of the microRNA,^{1,2} therefore, we assessed translation efficiency in distinct stages of the cell cycle by tethering analyses.

Here, we used the AGO2-tethered and control *prpΔ*-tethered reporters to examine translation when cells were arrested in G₁ by double thymidine block, in S phase by hydroxyurea treatment, and in S and G₂ phases upon release from double thymidine block.^{5,6} In each case, the cell cycle phase was verified by propidium iodide staining and FACS analysis (data not shown). While hydroxyurea treatment elicits strong translation repression by AGO2 (HU/S phase, Fig. 1A), similar to nocodazole treatment,² double thymidine block induces minimal repression (DT/G₁ phase in Fig. 1B). In contrast, cells released from double thymidine block that have progressed into the S and G₂ phases yield at least four-fold repression (RelDT/S phase and RelDT/G₂ phase, Fig. 1B). These data confirm our previous observations that in the G₁ phase of the cell cycle repression by AGO2 is poor (less than two-fold), while in the S and G₂ phases repression is strong (at least four-fold). The robust repression we previously reported in synchronized cultures² was most likely due to a more homogenous pool of S/G₂ cells, eliminating the problem of the diluting effect of cells in G₁ and/or G₀ phases in an asynchronous culture. It has been well established by FACS analyses that in asynchronous cultures of cell lines, two-thirds of the cells are in G₁, while the rest are primarily in S phase or to a lesser extent in the G₂ and M phases.⁷ This may in part explain some of the discrepancies in the literature regarding the magnitude of microRNA-induced translation repression in asynchronous cultures.

We next reexamined the question of whether translation activation is a consequence of serum-starvation-induced stress or also occurs during naturally-induced quiescence. We previously reported that activation can be observed with the ARE reporter not only by serum starvation but also by growing the cells to confluency in serum, which induces cell cycle arrest in G₀ and excludes possible stress influences from serum-starvation.¹ To demonstrate that activation by microRNAs can be achieved in natural G₀ quiescent conditions in a non-transformed cell line, we used the well-known NIH3T3 cell line, which enters G₀ upon contact inhibition.⁸ NIH3T3 cells were transfected with either the miRcxcr4 responsive CX reporter² or with the Let-7 responsive HMGA2 reporter⁹ in the presence and absence of additional miRcxcr4 or Let-7 microRNA, respectively (Fig. 2). The cells were grown to confluency and assessed 36, 48 and 72 hours later (Materials and Methods), when markers for G₀ arrest—maximal p27 upregulation (Fig. 2A) and a predominant, single G₁/G₀ peak upon FACS/propidium iodide analysis (data not shown)⁶—are observed. As expected, 48 hours after contact inhibition-induced G₀ conditions, the CX reporter (Fig. 2B) responded to addition of miRcxcr4 with about six-fold translation activation relative to no miRNA (control). Comparable stimulation was observed for the HMGA2 reporter in the presence compared to the absence of added Let-7 (Fig. 2C), while the mutant HMGA2 control reporter did not change significantly except in response to

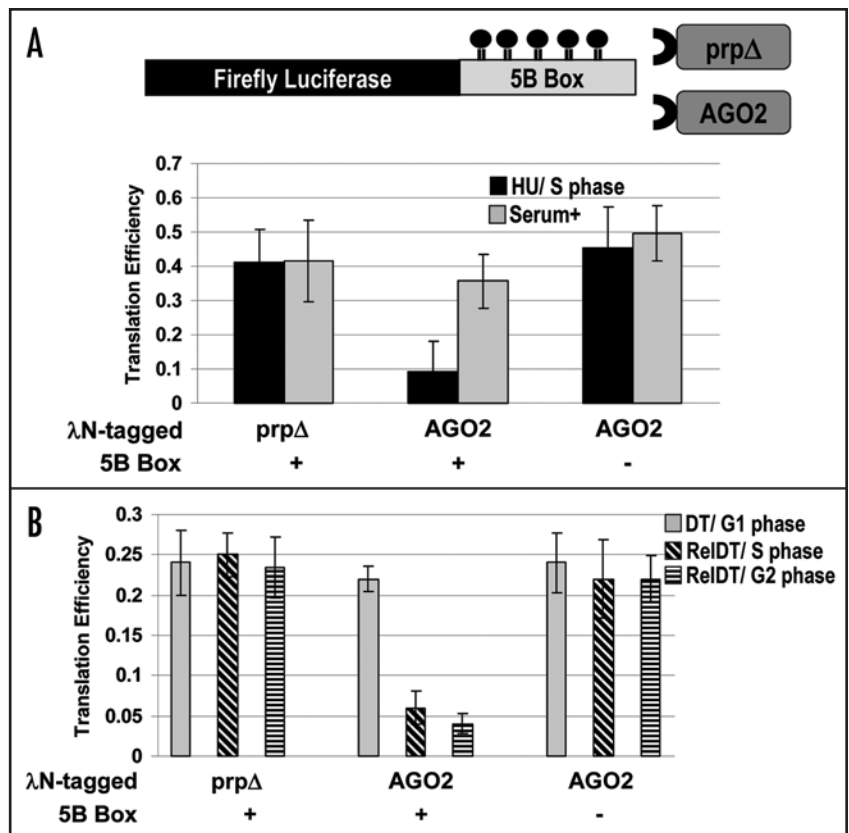


Figure 1. The S and S/G₂ phases of the cell cycle mediate significant microRNA-regulated translation repression while in the G₁ phase repression is minimal. Translation efficiency was analyzed with AGO2 or the inactive AGO2 mutant, *prpΔ*, both tagged with λN (depicted as a semi-circle on the proteins) tethered to a Firefly luciferase reporter bearing five B-box elements in its 3'UTR (depicted as hairpins).¹ HeLa cells were either grown asynchronously [denoted Serum⁺ in (A)] or were (A) synchronized in the S phase by hydroxyurea (HU/S phase) treatment (Materials and Methods) or (B) synchronized in G₁ phase by double thymidine block (DT/G₁), followed by release into fresh media to allow progression into the S and G₂ phases (RelDT/S phase and RelDT/G₂ phase, Materials and Methods).

a correspondingly mutated mtLet-7 microRNA that restored base-pairing.^{2,9} These data confirm that quiescence achieved by contact inhibition induces microRNA-mediated translation activation in the NIH3T3 cell line.

Finally, we investigated whether siRNAs, which by definition are perfectly complementary to their target mRNAs, can cause translation activation in response to serum-starvation-induced growth arrest. Our previous data suggested that siRNA knockdown is equally effective in arrested and growing cells,² indicating that siRNP activity is not regulated by growth arrest. We assayed two similar reporters, 4P and CX, both targeted by miRcxcr4, where 4P bears perfectly complementary sites and CX bears sites predicted to yield bulged pairing with the miRNA (Fig. 3, Doench et al.,¹⁰ and Doench and Sharp¹¹). With 4P, under either growth-arrested serum-starvation conditions or in growing cells synchronized in S/G₂, inhibition of Firefly luciferase activity was observed upon addition of miRcxcr4 (Fig. 3). In contrast, CX exhibited regulated expression in response to miRcxcr4, showing translation activation in the growth-arrested conditions and translation repression in the synchronized S/G₂ cells. We conclude that siRNPs and microRNPs are regulated differently by cell cycle conditions.

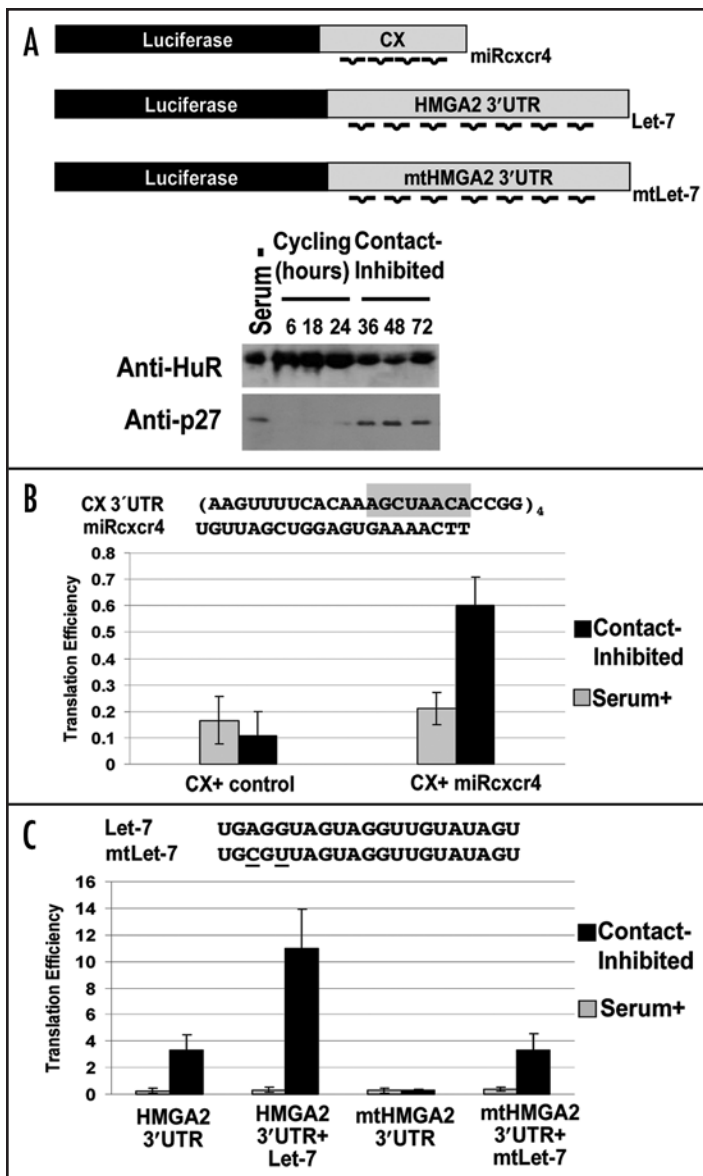


Figure 2. MicroRNAs mediate translation upregulation in naturally quiescent conditions in contact-inhibited NIH3T3 cells. NIH3T3 cells were grown asynchronously [denoted Serum⁺ in (B and C)] or were permitted to grow to contact inhibition as described.^{6,8} (A) Growth arrest upon contact inhibition or serum-starvation (Serum⁻) was verified by the accumulation of p27 by western blot analysis.⁷ HuR provided a control. (B) Translation efficiency was measured as in Figure 1 for the synthetic microRNA reporter CX (as described in Vasudevan et al.²) without and with addition of 50 nM miRxcrcr4 (Materials and Methods). (C) Reporters containing the 3'UTR of the Let-7 natural target HMGA2 wild-type and a mutant (mtHMGA2) mRNA were assayed without and with addition of 50 nM Let-7 or corresponding mutant Let-7 microRNA.

Discussion

Here, we have expanded our previous observations and provide further evidence that when cells withdraw from proliferation (G_0 or G_1/G_0 arrest), microRNA activity switches from translation repression to translation activation. Cells that have exited the cell cycle at the G_1 checkpoint and entered G_0 are said to be quiescent.^{7,12} Quiescence includes an assortment of distinguishable cell states uniquely characterized by the triggering signal; however, they

share a common quiescence program.¹² The expression of genes in an active quiescence program not only serves to maintain cell cycle arrest but also constitutes an active block to other alternative cell fates such as differentiation and apoptosis. Quiescence is the reversible alternative to proliferation; therefore, quiescent cells retain the ability to return to proliferation, unlike most terminally-differentiated cells. For instance, many immune cells, such as quiescent or anergic T cells and resting B cells, are quiescent as an important feature of their fate.^{13,14} Indeed, about 95% of hematopoietic stem cells are quiescent in vivo.^{15,16} It remains to be investigated whether specific cell fates and differentiation programs in various cell types show translation activation or repression by microRNAs.

The kinetics of return from G_0 /quiescence to the cell cycle are usually extended, leading to a lengthy dormant state called G_1pm (G_1 post-mitotic). G_1pm represents a step forward from true quiescence in which the cells are still uncommitted to the cell cycle, p27 is still present and the cells respond to signaling.⁶ G_1pm is considered an early G_1 state prior to the restriction point. Thereafter, cells become committed to true growth and macromolecular expansion in the G_1 phase (also called G_1ps for G_1 pre-synthesis) and continue into the subsequent DNA synthesis/S phase of the cell cycle.¹⁷⁻¹⁹ For example, B and T cells often show a long lag before proceeding to post-restriction G_1/G_1ps , during which they do respond to signaling but are not committed to the cell cycle; such cells do not enter S phase or cycle.²⁰⁻²² In the G_1/G_1ps phase, we observe poor translation repression (Fig. 1B, DT/ G_1 phase, double thymidine block treatment) and no translation activation, distinguishing this phase from the quiescent states of G_0 and G_1pm . It remains to be ascertained whether translation activation in different cell types is a function of true G_0 only or of G_1pm as well.

A few well-studied mRNAs such as TNF α (data not shown) exhibit a correlation between their protein expression in cell lines and activation by microRNAs. A study concurrent with our previous work² demonstrated that exogenous expression of miR155 upregulated a TNF α 3'UTR reporter directly or indirectly in HEK293 cells.²³ However, it remains to be determined if regulation also occurs in specific tissues according to their cell cycle states in an animal model. Additional influences such as chromatin and transcriptional regulation in vivo may modulate the regulatory potential of microRNAs on translation.

While siRNAs and microRNAs presumably associate with the same proteins, they elicit distinct outcomes. Specifically, siRNPs decrease expression by initiating mRNA degradation while microRNPs regulate both translation and mRNA decay.²⁴ Presently, it is unclear why microRNP activity responds to the cell cycle, whereas siRNPs do not (Fig. 3).

Our results suggest the potential for microRNAs to perform novel functions in distinct cellular conditions. The simplest model is that microRNAs activate translation in quiescent, arrested cells and repress translation in the later stages of the cell cycle (Figs. 1–3). This scenario correlates well with previous observations that GW bodies, which are essential for microRNA silencing, grow from fewer and smaller foci in the G_1 phase to larger and more numerous entities in the S phase, followed by an even greater increase in G_2 .²⁵ Thus, microRNA functions that are regulated by GW bodies would be subject to cell cycle regulation. However, additional novel functions

Figure 3. MicroRNAs and siRNAs are differentially regulated by the cell cycle: downregulation by siRNAs is observed in both proliferating cells and in serum-starved arrested cells, while microRNAs switch to translation activation in the latter condition. Two Firefly luciferase reporters, 4P and CX, with similar 3'UTRs were constructed; in 4P, the target sites are completely complementary to synthetic microRNA miRcxcr4 (depicted by straight lines parallel to the 4P 3'UTR) creating an siRNA reporter, while in CX (Fig. 2 and according to Vasudevan et al.²) the target sites are imperfectly paired with miRcxcr4 (depicted as bulged lines parallel to the CX 3'UTR) to create a microRNA reporter. The reporters were tested without or with the expression of miRcxcr4 (Materials and Methods) in either serum-starved arrested cells (Serum-) or in S/G₂ synchronized proliferating cells (Snc).

for these small regulatory RNAs may exist.

Why isn't all gene expression activated in a quiescent cell? It is evident from emerging literature that the 3'UTRs of mRNAs are not under the control of a single microRNA species even though a single microRNA may dominate under specific conditions. Protein binding sites or other regulatory sequences within the 3'UTR may influence a specific microRNA interaction even when the minimal microRNA target site in a reporter exhibits the predicted regulated phenotype.^{3,26} The overall gene expression outcome produced by a 3'UTR therefore likely reflects the sum of effects of selected control elements that are permitted to dominate in a given cellular condition.

Materials and Methods

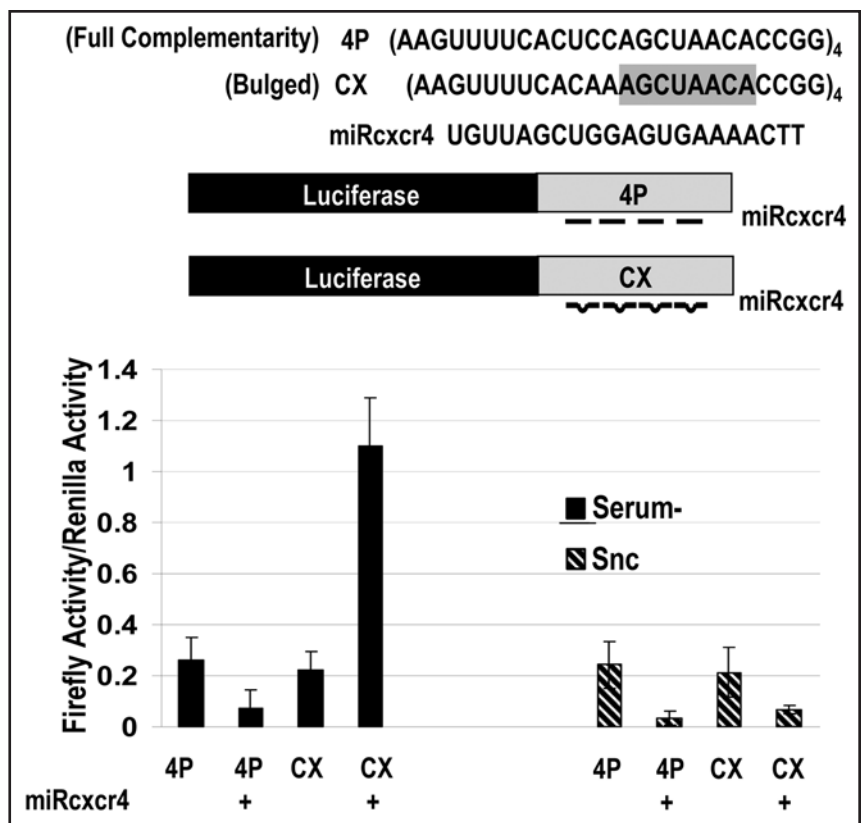
Cell culture. HeLa cells, HEK293 and HEK293 Tet-on cells were grown in DMEM + L-glutamine + Penicillin/Streptomycin (DMEM) with or without 10% fetal bovine serum (FBS). For NIH3T3 cells, the above media with 10% newborn calf serum (instead of FBS) was used.

Plasmids. CX and 4P are Firefly luciferase reporters constructed by inserting oligonucleotide sequences from Doench et al.,¹⁰ and Doench and Sharp¹¹ into a Not I site in the 3'UTR of the CTRL reporter.^{1,2} miRcxcr4 was expressed as an shRNA by cloning miRcxcr4 sequences from Doench and Sharp¹¹ into a tetracycline-inducible shRNA vector from Dickins et al.²⁷ HEK293 Tet-on cells were used for experiments with 4P or CX with 0.5 µg/ml doxycycline added to induce miRcxcr4. Alternatively, synthetic miRcxcr4 was added to NIH3T3 cells transfected with CX as described in Vasudevan et al.² All other plasmids were described earlier.^{1,2}

Cell transfection. Multiple methods of transfection (mild Mirus reagents, electroporation, nucleofection) follow the manufacturer's protocols and yield both repression and activation with microRNA test reporters.² Lipofectamine was avoided because it causes excessive cell death and prevents activation.² Transfection was performed as described below in the synchronization protocol.

Synchronization protocol. (1) HeLa cells are grown to low confluency (30–40%) by plating 10 mls of 20,000 cells/ml in a 25 cm-square flask and incubating for 24 hours at 37°C.

(2) In all experiments, transfection is performed with EndoGo (Mirus Bio) endotoxin-cleaned plasmids. This is essential as endotoxin inhibits activation; no greater than a two-fold stimulation can be obtained without it.



(3) Transfection using Mirus reagents, electroporation or nucleofection, following the manufacturer's protocol, yield both repression and activation with microRNA test reporters.² Only for transfection with lipofectamine, the cells are grown to 60–80% confluency before transfection to obtain repression. Lipofectamine works very well for repression because cells that are in G₂, where we see the strongest repression, are most efficiently transfected.²⁸ We do not see activation with lipofectamine, as described in Vasudevan and Steitz.¹

(4) 50–100 ng plasmid is transfected into 200,000 cells, preventing overexpression of the reporter mRNA in comparison to microRNA levels.

(5) Cells are exposed to the transfection complexes for 12–18 hours.

(6) If the cells are confluent or touching each other after exposing the cells to the transfection complexes for 12–18 hours, they must be diluted first for yet another 18–24 hours to produce a lower confluency where they are not touching each other. This is essential as serum-starvation-induced growth arrest will not be achieved otherwise.^{5,6}

(7) The cells are washed well—up to three times—with phosphate buffered saline (PBS). Then, serum-minus medium (DMEM) is added. (Washing out the serum thoroughly is necessary to ensure proper arrest.)

(8) The cells are incubated in serum-minus medium for at least 18 and no more than 36 hours. This allows the cells to completely deplete the serum in/on the cells. Some cell lines are viable for longer periods, while others undergo cell death after 36 hours. Several types of suspension cell lines contain a greater fraction of cycling cells as they are unanchored. When serum starved, they rapidly adhere and maintain adherence for about 3–4 hours, which is sufficient to see translation upregulation.

(9) Serum-plus medium (DMEM + 10% FBS) is added for another 18 hours to serum-starved cells to obtain synchronized cycling cells (where repression is strongest). Different HeLa cell lines have different cycling times and this synchronization time needs to be determined if the cells appear to double in less than 21 hours, which is normal for many HeLa and HEK293 lines.

(10) In each experiment, a parallel set of cells is checked by FACS/propidium iodide staining analysis or by p27 western blotting.^{5,6} A single dominant G₁ peak (G₀) is obtained for quiescent cells, as opposed to the normal G₁ and G₂ peaks observed for cycling cells; increased p27 levels confirm G₀ arrest.

(11) For growth to confluency or contact inhibition of cells such as the NIH3T3 cells, the cells are grown until the plate is confluent, then further incubated for another 36–48 hours to ensure complete arrest upon contact inhibition before analysis.^{6,8} As a control, one set of confluent cells is trypsinized and replated into a much larger flask/volume to restore 30–40% confluency for 6 hours; these cells demonstrate basal translation.¹ HeLa cell lines were observed to undergo cell death upon growth to complete confluency.

(12) To obtain repression in either the S or late S/G₂ phase, cells are released from double thymidine block or arrested directly in these phases by drug treatment; the phase is checked by FACS/propidium iodide staining analysis. Double thymidine block is accomplished by adding 2 mM thymidine to 30–40% confluent HeLa cells for 19 hours followed by release into thymidine-free medium for 9 hours followed by 2 mM thymidine treatment for 16 hours. Such cells can be maintained arrested in G₁ or released into thymidine-free medium for 3 hours (S phase) or 9 hours (G₂ phase) as analyzed by FACS/propidium iodide staining of parallel batches of cells. Alternatively, 2 mM hydroxyurea is added to cells for 12 hours to synchronize cells in the S phase. 400 ng/ml of nocodazole is added to cells for 14 hours to synchronize cells in the G₂ phase.^{2,5}

Luciferase assay. Extract preparation and luciferase assays are performed as described in the manufacturer's protocol (Promega). Each experiment is normalized to the Firefly and Renilla RNA levels to correct for slight variations in transfection/expression.¹

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