

fluorescent protein (GFP) tag on H4] (Fig. 3B). At later time points, the majority of GFP-positive cells arrested in late S/G<sub>2</sub>. We focused on the S-phase defect to address whether H3-H4 excess mimicked Asf1 depletion. The moderate increase in H3-H4 expression did not cause DNA damage monitored by  $\gamma$ -H2AX (fig. S8B). We thus analyzed RPA and PCNA profiles using GFP-negative cells (no H3.1-H4 induction) as an internal control for proper localization (Fig. 3C). Again, as in Asf1-depleted cells, RPA replication patterns in histone-over-expressing cells were barely visible, with some RPA localized to bright nuclear foci mainly corresponding to PML bodies (Fig. 3C and fig. S8D). Furthermore, as for Asf1 knockdown, an excess of new H3-H4 histones impaired ssDNA formation and RPA accumulation at replication sites (fig. S9, A and B), as well as checkpoint activation in response to HU (figs. S9C and S8C). Together, these data indicate that overproduction of histone H3-H4 impairs replication by impeding DNA unwinding. Consistent with the possibility that this results from interference with Asf1 function, we found that ectopic expression of Asf1a partially alleviated the inhibitory effect of histone excess on S-phase progression (Fig. 3D). Moreover, Asf1 depletion aggravated the S-phase defect resulting from histone H3-H4 excess, in that progression into G<sub>2</sub> was delayed even further (Fig. 3E).

Together, our results show that replication fork progression is dependent on the histone H3-H4 chaperone, Asf1, and on an equilibrium between histone supply and demand. This dependency could ensure that replication only proceeds when nucleosomes are being formed behind the fork with a proper balance between new and parental histones H3-H4. In the most parsimonious view, we propose a model (Fig. 4) in which Asf1 uses its properties as a histone acceptor and donor to facilitate unwinding

of the parental chromatin template in coordination with nucleosome assembly on daughter strands. Nucleosome disruption during replication fork passage would involve the histone-binding capacity of the MCM2–7 complex and transfer of parental histones to Asf1 through the Asf1–(H3-H4)–MCM intermediate, followed by their deposition onto daughter strands. In parallel, Asf1 would provide the additional complement of histones through its established role as a new histone donor (4, 20, 21). Asf1 knockdown will impair histone transfer and disruption of parental nucleosomes that thus present an impediment to unwinding and replication fork progression. Similarly, because of the dual function of Asf1, an excess of new histones will not leave Asf1 available for parental transfer, which impairs unwinding. On the basis of structural data (7, 22, 23), our model implies that parental histones (H3-H4)<sub>2</sub>, like new histones (24), go through a transient dimeric state during transfer. Furthermore, the MCM–(H3-H4)–Asf1 connection opens new angles to understand MCM2–7 function in chromatin (25). In conclusion, having Asf1 deal with both new and parental histones could provide an ideal means to fine-tune de novo deposition and recycling with replication fork progression. By offering a mechanism to coordinate new and parental histones during replication, our model should pave the way to addressing key questions regarding chromatin-based inheritance, including transmission of histone modifications.

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/318/5858/1928/DC1  
Materials and Methods  
Figs. S1 to S9  
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## Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation

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AU-rich elements (AREs) and microRNA target sites are conserved sequences in messenger RNA (mRNA) 3' untranslated regions (3'UTRs) that control gene expression posttranscriptionally. Upon cell cycle arrest, the ARE in tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) mRNA is transformed into a translation activation signal, recruiting Argonaute (AGO) and fragile X mental retardation-related protein 1 (FXR1), factors associated with micro-ribonucleoproteins (microRNPs). We show that human microRNA miR369-3 directs association of these proteins with the AREs to activate translation. Furthermore, we document that two well-studied microRNAs—Let-7 and the synthetic microRNA miRcxcr4—likewise induce translation up-regulation of target mRNAs on cell cycle arrest, yet they repress translation in proliferating cells. Thus, activation is a common function of microRNPs on cell cycle arrest. We propose that translation regulation by microRNPs oscillates between repression and activation during the cell cycle.

**A**U-rich elements (AREs) bind specific proteins to regulate mRNA stability or translation in response to external and internal stimuli (1). MicroRNAs are small non-

coding RNAs that recruit an Argonaute (AGO) protein complex to a complementary target mRNA, which results in translation repression or degradation of the mRNA (2, 3). We previously dem-

onstrated that the tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) ARE can be transformed by serum starvation, which arrests the cell cycle, into a translation activation signal (4). AGO2 and fragile X mental retardation-related protein 1 (FXR1) associate with the ARE on translation activation; both proteins are required to increase translation efficiency. Two key questions arose. First, is binding of the AGO2-FXR1 complex, which activates translation, directed by a microRNA complementary to the ARE? Second, can micro-ribonucleoproteins (microRNPs), in general, up-regulate translation under growth-arrest conditions, thereby switching between repressing and activating roles in response to the cell cycle?

A bioinformatic screen identified five microRNAs in miRBASE with seed regions complementary to the TNF $\alpha$  ARE, not including miR16 (5) [see supporting online material (SOM) text]. Of these, only human miR369-3 (Fig. 1A and fig. S1) tested positive in the following assays. Its seed sequence potentially forms base pairs with two target sites [seed1 and seed2, shaded in (Fig. 1A)] within the minimal TNF $\alpha$  ARE needed for translation activa-

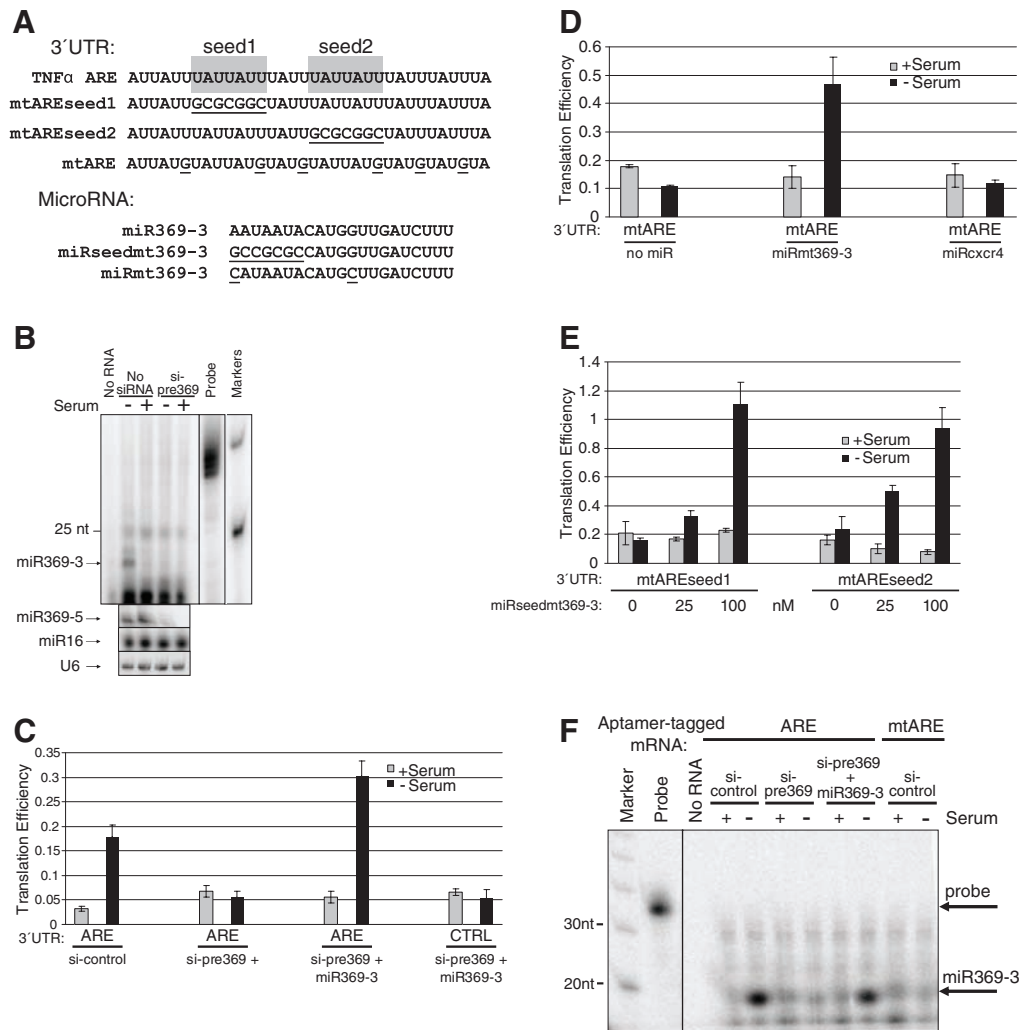
tion of the luciferase reporter (4). We find that expression of miR369-3 in HEK293 cells is reduced in serum-grown cells (Fig. 1B) and that miR369-3 is necessary for translation up-regulation of the ARE reporter. A small interfering RNA (siRNA) directed against the loop region of the miR369-3 precursor (si-pre369) (Fig. 1B and fig. S2) prevented translation (knocked down) up-regulation under serum-starved conditions. When knock-down was followed by rescue with synthetic miR369-3 resistant to the siRNA (Fig. 1C), translation efficiency was increased fivefold. These results indicate that miR369-3 is specifically required directly or indirectly for TNF $\alpha$  ARE-mediated translation activation under growth-arrest conditions.

To test whether translation activation requires formation of base pairs between miR369-3 and the ARE, we used a mutant ARE (mtARE) (4) that

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**Fig. 1.** MiR369-3 is required to activate TNF $\alpha$  ARE reporter translation under growth-arrest conditions. **(A)** 3'UTR sequences of the luciferase reporter: wild-type TNF $\alpha$  ARE and mutants mtAREseed1, mtAREseed2, and mtARE (AUUUAs converted to AUGUAs). MicroRNA sequences: wild-type miR369-3, seed mutant miRseedmt369-3 (complementary to mtAREseed1 and mtAREseed2), and miRmt369-3 (complementary to mtARE). Target seed regions are shaded; mutations are underlined. **(B)** Ribonuclease protection assay (RPA) for miR369-3 in serum-starved (-) and serum-grown (+) HEK293 cells without or with siRNA treatment against pre-miR369 (si-pre369). MiR369-5 (fig. S2), miR16, and U6 RNA provided controls. **(C)** All translation assays (4) used firefly luciferase reporters [here ARE or control (CTRL)]; values were normalized to a *Renilla* cotransfected reporter and to the firefly and *Renilla* mRNA levels to determine translation efficiency (5). HEK293 cells grown in serum (+) or without serum (-) were transfected with 50 nM miRcxr4 (si-control, Fig. 2A), si-pre369 (fig. S2), or si-pre369 plus synthetic miR369-3. **(D)** Translation efficiency of the mtARE reporter without added microRNA or with 50 nM miRmt369-3 or miRcxr4 control. **(E)** Translation efficiency in serum-grown or -starved cells transfected with either the mtAREseed1 or mtAREseed2 reporter without or with miRseedmt369-3. **(C)**, **(D)**, and **(E)** show averages from at least three transfections  $\pm$  standard deviation. **(F)** S1-aptamer-tagged ARE and mtARE reporters were used for in vivo cross-linking-coupled RNP purification (4) from HEK293 cells grown in serum under basal translation conditions (+) or serum-starved translation activation conditions (-). MiR369-3 was detected by RPA.



does not undergo translation up-regulation in either serum condition. We added miR369-3 mutated to restore complementarity (miRmt369-3) (Fig. 1A and fig. S1) and observed that translation is up-regulated only under serum-starved conditions (Fig. 1D). Next, we introduced identical mutations into the ARE at each site complementary to miR369-3's seed region (Fig. 1A and fig. S1) and found that both mtAREseed1 and mtAREseed2 exhibited loss of translation up-regulation, which could be restored by adding increasing amounts of seed-mutated miRseedmt369-3 (Fig. 1E). Because endogenous wild-type miR369-3 is also present, this suggests that both sites are required to form base pairs with miR369-3. Finally, to confirm the involvement of miR369-3, we probed affinity-purified ARE mRNA after formaldehyde cross-linking to preserve in vivo RNP complexes (4). Fig. 1F reveals enhanced levels of miR369-3 in the ARE relative to the mtARE-containing complex from serum-starved (-) but not serum-grown (+) cells or when miR369-3 was added to rescue the knockdown of microRNA by si-pre369. Together, these results indicate that miR369-3 associates with the TNF $\alpha$  ARE to up-regulate translation exclusively upon serum starva-

tion by direct base pairing between its seed sequence and complementary ARE regions.

Tethering AGO2 to a reporter mRNA under conditions of serum starvation up-regulates translation (4), which suggests that—like miR369-3 recruitment to an ARE (Fig. 1)—other microRNPs might be transformed into activating complexes on cell cycle arrest. This hypothesis was tested by creating a reporter with four artificial 3'UTR target sites (CX) (6, 7) (Fig. 2A). In response to its corresponding synthetic microRNA (miRcxr4) (6, 7) added exogenously, CX mRNA translation was up-regulated exclusively upon serum starvation (Fig. 2A). No difference in CX translation with or without serum in the absence of added microRNA indicates that the reporter assay behaves comparably under different growing conditions (SOM text). We extended these analyses to high-mobility group A2 (HMGA2) 3'UTR reporter, which contains seven sites for the endogenous Let-7 microRNA, and a matching control with mutated sites (8) (Fig. 2B). When HeLa cells were serum-starved, as opposed to grown in the presence of serum (+Snc), HMGA2 translation efficiency increased significantly and was further amplified by addition of

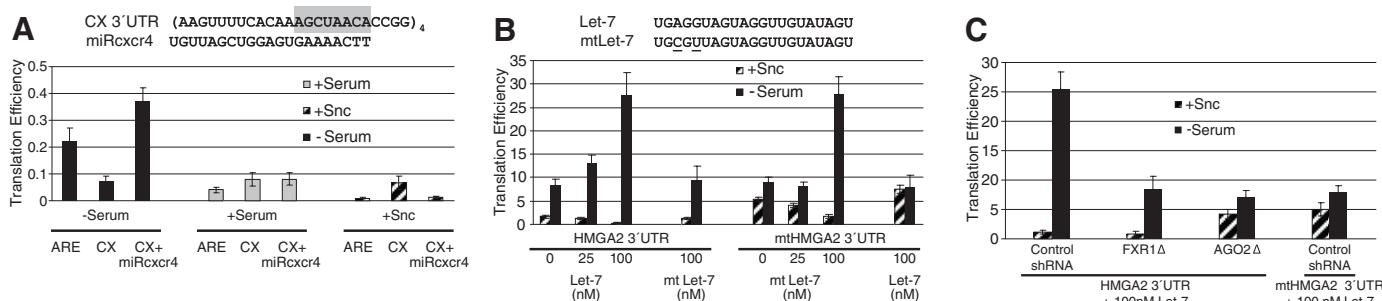
exogenous Let-7 (Fig. 2B). A requirement for microRNA base-pairing was demonstrated using the mutant HMGA2 reporter with and without exogenous complementary mutated Let-7 (Fig. 2B, black bars). Knockdown experiments showed that HMGA2 translation up-regulation is also dependent on the presence of FXR1 and AGO2 (4) (Fig. 2C). We conclude that all three microRNAs studied switch to translation activation under growth-arrest conditions.

In both HEK293 and HeLa cells, serum starvation causes translation activation relative to basal levels, which is distinct from alleviating translation repression to restore basal levels. We define basal as the translation efficiency [normalized luciferase activity (see tables S1 to S8)] of reporters that do not contain ARE or of a microRNA-targeted reporter in the absence of the corresponding microRNA (Figs. 1 and 2A), because these values are similar in all tested conditions. Increased translation efficiency is considered activa-

tion, whereas translation below this reference level constitutes repression. In serum-grown asynchronous cells, basal translation was observed not only for the ARE, as expected from the lack of miR369-3 (Fig. 1B), but also for the CX reporter in the presence of miRcxcr4 (Fig. 2A, gray bars), which should experience repression according to the literature (6). Because translation activation is controlled by the cell cycle (4), we reasoned that careful synchronization of the entire cell population might allow translation repression to be better distinguished from basal translation (5). Therefore, we subjected HeLa cells to serum starvation followed by release into serum growth conditions for 18 hours, which results in synchronous proliferation (in late S/G<sub>2</sub> phase) (5). Translation in these synchronized conditions (+Snc) was significantly repressed (lower than basal translation in asynchronous cells grown in serum by a factor of 3 to 5) for both the ARE reporter and the CX reporter cotransfected with miRcxcr4 (Fig. 2A,

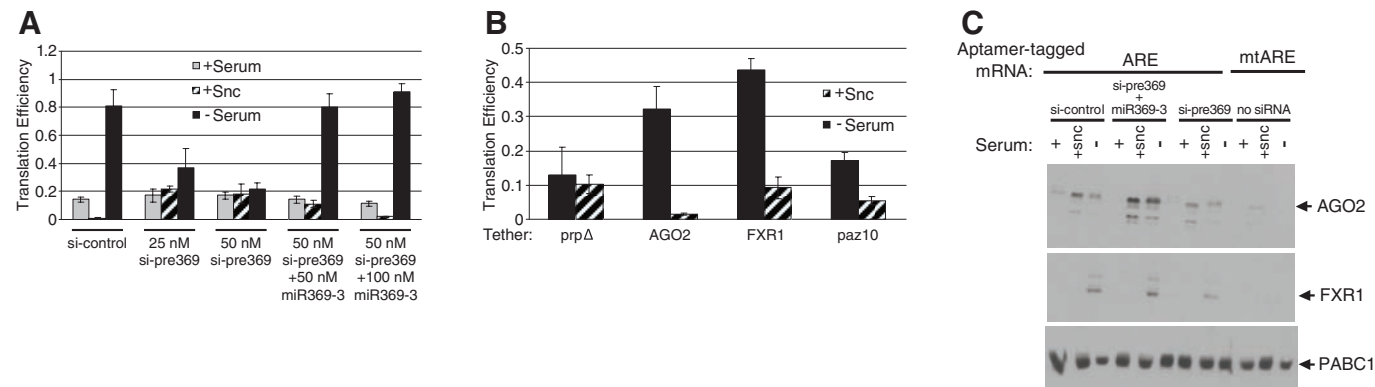
striped bars). Synchronization also produced significant repression upon cotransfection of the natural microRNA-targeted HMGA2 3'UTR reporter and exogenous Let-7 or of the mutated HMGA2 reporter and the corresponding mutated Let-7 (Fig. 2B, striped bars) (8). Such repressive effects were reproduced with the ARE reporter under synchronized growth; knockdown of the miR369-3 precursor alleviated translation repression, whereas adding back synthetic miR369-3 reversed repression (Fig. 3A, striped bars).

Under synchronized proliferation conditions, tethering λN-tagged AGO2 fusion protein to the 5B box reporter (4, 9) revealed translation repression by a factor of five (Fig. 3B) and a comparable extent of activation (4) under growth-arrested conditions, relative to mutant λN-tagged AGO2 proteins (ppΔ and paz10, respectively) (9, 10). The cell cycle regulation of translation was further established by treatment with aphidicolin (which causes G<sub>1</sub> arrest), which led to translation activa-



**Fig. 2.** Two other microRNAs switch from effecting translation activation under cell cycle-arrested conditions to repression in proliferating cells. (A) Translation of the ARE or CX reporter, with four tandem copies of the sequence complementary to the artificial *cxcr4* target site (6, 7), was assessed as in Fig. 1, C to E, in HeLa cells transfected without or with addition of miRcxcr4 under three different conditions: Black bars are cells grown without serum (translation up-regulation), gray bars are asynchronous cells grown in serum (basal translation) (4), and striped bars are serum-grown cells synchronized by release from serum starvation (+Snc) (translation repression). Cells

cotransfected with control miRs (mtLet-7) gave results comparable to ARE or CX alone (not shown). (B) Translation efficiency in cells transfected with the wild-type HMGA2 3'UTR or mtHMGA2 3'UTR *Renilla* reporter (8) without or with increasing concentrations of Let-7 or the compensatory mtLet-7 miRNA (8) and under synchronized or serum-starved conditions. (C) Translation efficiency of cells transfected as in (B), but with prior RNA interference treatment with short hairpin RNA to knockdown FXR1 or AGO2 as described (4). (A), (B), and (C) show averages from at least three transfections ± standard deviation.



**Fig. 3.** MicroRNA and protein requirements for translation activation and repression. (A) Translation efficiency of cells transfected with the ARE reporter grown under the three conditions described in Fig. 2A, without or with increasing concentrations of si-pre369 and synthetic miR369-3 to rescue the knockdown. (B) Translation efficiency in cells cotransfected with the 5B box reporter (4) and various λN-tagged proteins: ppΔ (9), AGO2, FXR1 (4), or paz10 (10) under synchronized or serum-starved conditions. (A) and (B) show averages from at least three transfections ± standard deviation. (C) Eluates from

S1-aptamer-tagged ARE and mtARE reporters after RNP purification (as in Fig. 1F) were subjected to Western analyses for AGO2, FXR1, and PABC1 (as a control). The lower band in the gel probed with antibody against AGO2 is a degradation product. In the three conditions, cellular AGO2 levels are unchanged, and miR369-3 can be immunoprecipitated by AGO2-specific antibody (not shown; see fig. S4 for levels of miR369-3 in synchronized cells). FXR1 usually runs as multiple bands that represent either multiple isoforms or modifications (4).

tion, or nocodazole (which arrests cells in G<sub>2</sub>/M), which led to translation repression by tethered AGO2 but not by the paz10 or prpΔ mutant AGO protein (fig. S3). Tethering FXR1 failed to cause translation repression (Fig. 3B), possibly because FXR1 is part of the activating but not the repressive AGO2 microRNP. Furthermore, the AGO2 mutant (paz10) that does not bind microRNAs is compromised in translation activation, which indicates a requirement for microRNA binding or that the PAZ-microRNA binding domain has additional roles (10).

To confirm that miR369-3 recruits the AGO2-FXR1 complex for translation up-regulation, we performed RNP purification of the aptamer-tagged ARE and mtARE reporters (4) from cells transfected with a control siRNA, si-pre369, or si-pre369 with synthetic miR369-3. Control siRNA-treated samples showed the presence of AGO2 and FXR1 (Fig. 3C), in addition to miR369-3 (Fig. 1F), in the ARE complex under serum-starved (-) conditions. Knockdown of miR369-3 production reproducibly reduced AGO2 and FXR1 on the ARE reporter, whereas rescue with synthetic miR369-3 restored AGO2-FXR1 to at least normal levels (Fig. 3C), which correlated with the translation efficiencies seen in Fig. 1C. We conclude that miR369-3 is required for recruitment of the AGO2-FXR1 complex, which activates translation, and that FXR1 is not part of the repressive complex (+Snc lanes in Fig. 3C).

Together our data indicate that microRNA repression is a property of proliferating cells. In the literature, the fold repression by AGO2 or microRNPs fluctuates, depending on the cell line, transfection method, and overall system (4, 9, 11, 12). Such variations may reflect differences in the cell cycle state of asynchronous populations and their response to transfection protocols.

We have shown that the TNF $\alpha$  ARE recruits miR369-3 to mediate translation up-regulation in serum-starved conditions and to cause repression in synchronized proliferating cells. Base-pairing of miR369-3 is required to recruit the activating AGO2-FXR1 complex (4). The TNF $\alpha$  ARE belongs to an ARE class typified by tandemly repeated AUUUA motifs (12, 13), but it contains three AUUA sequences, two with flanks that allow base-pairing with the seed region of miR369-3 (fig. S1). Both sites shaded in Fig. 1A appear to be required for microRNA-dependent translation activation (Fig. 1E), but their close spacing warrants further investigation (6, 14). Because AREs of the same class resemble each other but have distinct AU-rich sequences, they may recruit different microRNAs, which would explain their highly specific regulated expression patterns (12, 13).

MicroRNAs oscillate between repression and activation in coordination with the cell cycle: In proliferating cells they repress translation, whereas in G<sub>1</sub>/G<sub>0</sub> arrest (which often precedes differentiation), they mediate activation. This regulation occurs on at least two levels. First, recruitment of the microRNP reflects both its expression level and its ability to productively interact with mRNA

target sites. Second, the AGO2 complex must be subject to modification because tethered AGO2 differentially regulates translation according to cell growth conditions (Fig. 3B and fig. S3). As FXR1 is found exclusively in the activation complex and activation by AGO2 tethering in serum-starved conditions requires FXR1 expression, modifications that switch AGO2 from repressor to activator may alter interactions with FXR1. Such modifications upon serum starvation were suggested by changes in the solubility and subcellular localization of the AGO2-FXR1 complex (4). Our findings define a novel role for microRNAs and reveal an unanticipated versatility of microRNP function in response to the cell cycle, with important implications for understanding the contributions of these RNAs to development, differentiation, and carcinogenesis.

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#### Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S13

Tables S1 to S8

References

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## Rapid Changes in Throughput from Single Motor Cortex Neurons to Muscle Activity

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Motor cortex output is capable of considerable reorganization, which involves modulation of excitability within the cortex. Does such reorganization also involve changes beyond the cortex, at the level of throughput from single motor cortex neurons to muscle activity? We examined such throughput during a paradigm that provided incentive for enhancing functional connectivity from motor cortex neurons to muscles. Short-latency throughput from a recorded neuron to muscle activity not present during some behavioral epochs often appeared during others. Such changes in throughput could not always be attributed to a higher neuron firing rate, to more ongoing muscle activity, or to neuronal synchronization, indicating that reorganization of motor cortex output may involve rapid changes in functional connectivity from single motor cortex neurons to  $\alpha$ -motoneuron pools.

In a variety of situations, such as learning new fine motor skills, the human motor cortex can reorganize, increasing output to specific muscles (1, 2). Similarly, in monkeys that have learned movements, the territory from which intracortical microstimulation (ICMS) evokes output to trained muscles expands (3), and ICMS triggered from the voluntary discharges of flexion-related neurons can convert local output from excitation of extensor muscles to excitation of flexors (4). Does such plasticity of cortical output reflect changes in intracortical excitability exclusively, or can the throughput from single motor cortex neurons to muscles also change rapidly?

In two monkeys, we recorded neurons in the primary motor cortex (M1) hand representation

simultaneously with electromyographic (EMG) activity from up to 16 contralateral forearm and hand muscles. Each monkey performed two behavioral tasks: first, a simple hand squeeze task; second, a paradigm that provided incentive for the monkey to increase throughput from M1 neurons to muscles, which we term reinforcement of physiological discharge (RPD). Similar to previous studies (5–7), in RPD we rewarded the monkey for simultaneous ( $\pm 6$  ms) discharge of (i) spikes from an M1 neuron and (ii) large potentials in the EMG of a selected muscle, termed the RPD muscle. Typically, after recording a neuron during a squeeze task epoch, the same neuron was combined with different RPD muscles in successive epochs. For example, synchronous potentials from a given