Introduction

miRNAs are ~22 nucleotide (nt) sized gene regulators that have pervasive roles in development and disease [1,2]. They interact with argonaute (Ago) proteins [3] and guide RNA-induced silencing complexes (RISC or miRNP) to target mRNAs [1,2]. Binding of animal miRNAs to imperfectly matching sequences, usually in the 3’ untranslated region (UTR) of their target mRNAs [4], can inhibit accumulation of the encoded proteins by reducing mRNA translation and/or stability [5,6,7,8]. The relationship between miRNA-mediated deadenylation and translational repression has been less clear. Using transfection of reporter constructs carrying three imperfectly matching let-7 target sites in the 3’ UTR into mammalian cells we observe rapid target miRNA deadenylation that precedes measurable translational repression by endogenous let-7 miRNA. Depleting cells of the argonaute co-factors RCK or TNRC6A can impair let-7-mediated repression despite ongoing miRNA deadenylation, indicating that deadenylation alone is not sufficient to effect full repression. Nevertheless, the magnitude of translational repression by let-7 is diminished when the target reporter lacks a poly(A) tail. Employing an antisense strategy to block deadenylation of target mRNA with poly(A) tail also partially impairs translational repression. On the one hand, these experiments confirm that tail removal by deadenylation is not strictly required for translational repression. On the other hand they show directly that deadenylation can augment miRNA-mediated translational repression in mammalian cells beyond stimulating mRNA decay. Taken together with published work, these results suggest a dual role of deadenylation in miRNA function: it contributes to translational repression as well as mRNA decay and is thus critically involved in establishing the quantitatively appropriate physiological response to miRNAs.

Abstract

Animal microRNAs (miRNAs) typically regulate gene expression by binding to partially complementary target sites in the 3’ untranslated region (UTR) of messenger RNA (mRNA) reducing its translation and stability. They also commonly induce shortening of the mRNA 3’ poly(A) tail, which contributes to their mRNA decay promoting function. The relationship between miRNA-mediated deadenylation and translational repression has been less clear. Using transfection of reporter constructs carrying three imperfectly matching let-7 target sites in the 3’ UTR into mammalian cells we observe rapid target miRNA deadenylation that precedes measurable translational repression by endogenous let-7 miRNA. Depleting cells of the argonaute co-factors RCK or TNRC6A can impair let-7-mediated repression despite ongoing miRNA deadenylation, indicating that deadenylation alone is not sufficient to effect full repression. Nevertheless, the magnitude of translational repression by let-7 is diminished when the target reporter lacks a poly(A) tail. Employing an antisense strategy to block deadenylation of target mRNA with poly(A) tail also partially impairs translational repression. On the one hand, these experiments confirm that tail removal by deadenylation is not strictly required for translational repression. On the other hand they show directly that deadenylation can augment miRNA-mediated translational repression in mammalian cells beyond stimulating mRNA decay. Taken together with published work, these results suggest a dual role of deadenylation in miRNA function: it contributes to translational repression as well as mRNA decay and is thus critically involved in establishing the quantitatively appropriate physiological response to miRNAs.
m$^7$GpppN cap structure and the cap-binding eukaryotic initiation factor (eIF) 4E was impaired during miRNA-mediated repression. Our observations furthermore suggested that a miRNA inhibits poly(A) tail function during translation initiation [19], possibly by interfering with the formation of an mRNA closed-loop configuration, which is enabled by joint binding of eIF4E and the poly(A)-binding protein PABP to the bridging protein eIF4G [43,44,45]. In further support of this model, a poly(A) tail was required to demonstrate miRNA-mediated repression in two mammalian in vitro translation systems [20,25]. By contrast, a study examining repression by the endogenous let-7 miRNA family in HeLa cells reported no poly(A) tail dependence of translational repression [18]. Moreover, several reporter mRNAs lacking a poly(A) tail could still be silenced in mammalian and D. melanogaster cells, though in some cases at a reduced level [40,42,46]. Thus an emerging consensus is that the presence of a poly(A) tail on a target mRNA and/or its removal by deadenylation is not strictly required for miRNA-mediated translational repression, but to what extent it may still augment it is less clear.

This situation prompted us to further investigate the involvement of the poly(A) tail and its removal by deadenylation in miRNA-mediated translational repression. We report here that an established model of let-7-mediated repression in mammalian cells [18] features marked deadenylation that precedes the onset of observable translational repression and mRNA destabilisation. We find that depletion of argonaute co-factors can reduce let-7-mediated repression without noticeably affecting miRNA deadenylation, confirming that repression can be enacted by a deadenylation-independent mechanism. Nevertheless, we show that the magnitude of translational repression is reduced when the let-7-targeted reporter mRNA lacks a poly(A) tail, or when we block the deadenylation of a reporter mRNA with poly(A) tail. Our observations thus indicate that in mammalian cells deadenylation can contribute not only to miRNA decay but also translational repression mediated by an endogenous miRNA.

Results

Let-7 promotes target mRNA deadenylation

To further characterise the role of target mRNA deadenylation in the miRNA mechanism we employed an established model of let-7-mediated repression in mammalian cells [18]. Thus, we transfected HeLa cells with the plasmid pRL-3xbulge expressing a Renilla luciferase (R-luc-3xb) mRNA with three bulged let-7 sites in the 3’UTR region, together with the plasmid pGL3 expressing a Firefly luciferase mRNA (F-luc) as a transfection efficiency control. Parallel transfection of cells with the seed region-mutated let-7 anti-miR restores R-luc-3xb reporter expression to R-luc-mut levels on the protein as well as mRNA level (Fig. 1B). The let-7 anti-miR further selectively prevented R-luc-3xb mRNA deadenylation (Fig 1D). Collectively, these results show that this reporter system features let-7-specific translational repression, as well as moderate mRNA destabilisation as previously reported [18]. Importantly, they further show that it also displays let-7-mediated mRNA deadenylation.

Endogenous miRNA-targeted mRNAs accumulate with short poly(A) tails

We then asked if an endogenous let-7-targeted mRNA similarly undergoes deadenylation. We therefore performed LM-PAT assays for HMG A2 mRNA, a confirmed let-7 target [49,50]. We found in HeLa cells that HMG A2 mRNA is present primarily in an oligo-adenylated form, while transfection of let-7 anti-miR shifts it to a longer-tailed form (≥40 nt, Fig. 1D); this is blocked by actinomycin D treatment suggesting that the longer-tailed HMG A2 mRNA molecules arise from new transcription (data not shown). Conversely, we observed a shift from longer-tailed to oligo-adenylated HMG A2 during neuronal differentiation of mouse embryonic carcinoma P19 cells (Fig. 1E). We chose this cellular transition because it is known to entail a dramatic increase in let-7 levels [Fig. 1E] [51], mRNA deadenylation is emerging as a feature of miRNA action in different systems [38,39,40,41]. To extend observations in mammalian cells, we assessed the steady-state poly(A) tail length of seven validated miRNA targets [52,53] in cell lines reported to express both the mRNAs and cognate miRNAs [54]. Six of these miRNAs clearly accumulated oligo-adenylated forms, while the ACTB control mRNA does not (see Supporting Information S1 & Fig. S1). This cursory screen of additional endogenous miRNA targets is thus consistent with miRNA-mediated deadenylation being widespread in mammalian cells. This mirrors conclusions from recent microarray analyses in D. melanogaster, which showed that a widespread miRNA-mediated destabilisation of target mRNAs was dependent on the CCR4:NOT deadenylase complex [42]. Importantly, our data demonstrate let-7-dependent deadenylation of the cancer-related HMG A2 mRNA during a physiologically relevant cellular differentiation process as well as in the experimental conditions employed for our R-luc reporter studies.
Figure 1. mRNAs targeted by let-7 exhibit shortened poly(A) tails. (A) Schematic of R-luc constructs carrying three let-7 target sites in their 3' UTR (functional: R-luc-3xbulge; seed region mutated: R-luc-3xbulgemut [18]). (B) Let-7-dependent repression of R-luc protein and mRNA levels. HeLa cells were cotransfected with plasmids pR-luc-3xb or pR-luc-mut and pGL3 (F-luc) as control, and with an LNA anti-miR to let-7, miR-499 (non-specific; ns), or mock transfected (none) as indicated. Protein and RNA was extracted from cells at 6 and 24 hours after transfection. R-luc expression was normalised to F-luc expression for both protein and mRNA, measured by luciferase assay and qPCR, respectively. Repression by endogenous let-7 was calculated as the ratio of R-luc-mut expression to that of the corresponding R-luc-3xb. The bars represent averages of 2–3 measurements with standard error (24 hour protein) or range (all others). (C) Schematic of the LM-PAT assay and performance test. An LM-PAT assay for GAPDH mRNA was performed with HeLa cell total RNA (left panel). To mark the position of the shortest possible LM-PAT product, PCR was also carried out with cDNA primed with an anchor-(dT)12VN oligonucleotide that clamps to the 3' UTR–poly(A) junction (labeled 'TVN' above lane). An RNA sample was also subjected to RH-Hs GAPDH oligonucleotide-mediated cleavage by RNAse H and high resolution northern blot analysis (right panel). Cleavage was further done in the presence of oligo(dT) to generate a 3' UTR fragment without poly(A) tail. Position of size markers are indicated to the right of the panels (in base-pairs or nucleotides, respectively). (D) The poly(A) tail lengths of R-luc reporter and endogenous GAPDH or HMGA2 mRNAs were measured by LM-PAT assay six hours after transfection. (E) P19 cells were induced to differentiate by treatment with retinoic acid and RNA was purified from cells at the times indicated. Let-7 and U6 RNA (loading control) expression were measured by northern blotting (10 μg total RNA per lane), while HMGA2 and GAPDH mRNA poly(A) tail lengths were measured by LM-PAT assay.

doi:10.1371/journal.pone.0006783.g001
Let-7-mediated mRNA deadenylation is rapid

Next we explored the timing of miRNA-mediated deadenylation in relation to miRNA-directed translational repression, by performing a 48-hour time-course in HeLa cells transiently transfected with the let-7 reporter plasmids. While not being a strict pulse-chase scenario, this approach still featured a marked and transient burst of mRNA expression and likely mirrors the repression by a miRNA over the lifespan of an mRNA (see Fig. S2 for raw expression data). Between four and six hours, modest repression (~2-fold) of both R-luc protein and mRNA levels becomes apparent (Fig. 2A). Beyond 8 hours the levels of the let-7-regulated mRNA stabilize at ~2 to 3-fold less than control, but measured repression on the protein level continues to increase, reaching its maximal level (~11-fold) at 36 hours, an increase that cannot be attributed to mRNA stability decreases alone. LM-PAT assays were performed in parallel (Fig. 2B, C). As expected, R-luc-mut mRNA accumulates as long-tailed molecules (~150 nt of poly(A); up to eight hours) that become gradually deadenylated over time. Strikingly, while polyadenylated R-luc-3xb mRNA is initially detectable, it is already severely deadenylated three hours after transfection (Fig. 2C). These experiments indicate that miRNA-mediated deadenylation is a rapid step in the mechanism of miRNA action.

Let-7-mediated repression can be attenuated despite ongoing deadenylation

We furthermore depleted HeLa cells of the Ago co-repressors RCK/p54 or GW182 to explore the interdependence of let-7-mediated repression and deadenylation. We used proven siRNAs
against RCK/p54 or the TNRC6A paralog of GW182 [55], which led to efficient knock-down, while miCXCR4 [19,31] served as a non-targeting siRNA control in these experiments (Fig. 3A, B). Twenty-four hours after siRNA treatment, cells were transfected with let-7 reporter plasmids to measure effects on repression and deadenylation. The dual transfection regimen led to some variability in achievable repression in the non-specific control, as observed in previous reports [22,55]. Similar to these studies, we scaled the data within each set to the mock transfection (0% derepression, equivalent to an average reduction of ~2.5-fold on mRNA and ~5-fold on protein level), and let-7 anti-miR controls served as a reference for 100% derepression (see Fig. 1B), to account for this systemic variation in the set-point of repression. RCK or TNRC6A depletion both relieved let-7-mediated repression of R-luc protein and mRNA level (~75% derepression at 24 hours after plasmid transfection; Fig. 3C, D). We then measured let-7-mediated deadenylation by LM-PAT (Fig. 3E; six hours after plasmid transfection). This revealed no significant change in R-luc-3xb mRNA deadenylation with either RCK or TNRC6A depletion, or for control siRNA transfection. Minor differences in LM-PAT data between conditions discernible in Fig. 3E were due to the dual transfection regimen, but no consistent trend emerged in three to four independent biological repeats (data not shown) nor did this variability ever approach the distinct tail length stabilization induced by let-7 anti-miR treatment. We furthermore transfected in vitro transcribed capped R-luc-mut mRNA with or without a poly(A) tail into knock-down cells (Fig. 3F; see below for details), to test whether we inadvertently impared the poly(A) tail-dependence of cellular translation by depleting RCK or TNRC6A. This was not the case as each cellular condition gave similarly strong responses to the presence of a poly(A) tail (Fig. 3F).

RCK and GW182 both have reported roles in the miRNA mechanism [13,14,15,46,55,56,57,58,59]. Our finding that RCK depletion does not prevent let-7-mediated deadenylation is consistent with previous studies in D. melanogaster cells, where depletion of RCK/Mec31B, together with other decapping activators, led to accumulation of deadenylated miRNA-target mRNAs [60]. Work on human and D. melanogaster GW182 proteins showed that their silencing activity could in part be attributed to mRNA deadenylation [16,41]. However, GW182 proteins have repressive effects that are separable from their role in deadenylation and the situation in vertebrates is further complicated by ample opportunity for functional redundancy and specialisation among the three paralogs TNRC6A, B, and C [12,13,15,42,46]. Our observation that let-7-mediated deadenylation was impeded in cells depleted of TNRC6A may thus simply mean that we have not been able to reduce GW182 proteins below a required threshold level. With regard to the main objective of these experiments, we can nevertheless conclude that Ago co-factor depletion can cause significant relief of let-7-mediated repression despite ongoing target mRNA deadenylation, indicating that deadenylation alone is not sufficient to generate full repression.

Presence of a poly(A) tail enhances let-7-mediated repression

Next we directly examined a possible contribution of the poly(A) tail and its removal to translational repression by let-7. To this end we employed a direct mRNA transfection approach [18,19,48]. We prepared m′GpppG-capped R-luc-3xb and R-luc-mut mRNA in vitro (Fig. 4A, Fig. S3) with or without a poly(A) tail and transfected these into HeLa cells together with an F-luc control. Measurement of reporter protein levels, normalisation for transfection efficiency and calculation of repression by let-7 was done as for the plasmid transfection experiments described above. This showed that adding a poly(A) tail to R-luc-mut mRNA improved translation as expected (five to six-fold over cap alone, measured 16 hours after mRNA transfection; Fig. 4B, see also Fig. 3F). Repression by let-7 was significantly stronger for the cap&tail form of R-luc mRNA (~13-fold) than for capped mRNA (~6-fold repression; Fig. 4C). Similar differences in translational repression between cap and cap&tail forms of target mRNA were seen in undifferentiated P19 cells co-transfected with synthetic let-7 (Fig. S4). To determine the contribution of let-7-mediated mRNA destabilisation to repression, we measured R-luc mRNA levels recovered from transfected HeLa cells by real-time RT-PCR and found no significant differences between 3xb and mut mRNAs (data not shown). Given the possible complications with measuring physical mRNA stabilities in RNA-transfected cells [19,61], we also estimated functional mRNA half lives [62], by following the accumulation of R-luc protein over time (Fig. 4D, E). This revealed that R-luc 3xb mRNA was moderately less stable than the mut control (~1.4 fold for the cap&tail and ~1.3 fold for the cap only versions), confirming previous observations that let-7 mediated repression of transfected R-luc mRNA occurs mainly at the level of translation [18]. Collectively, these data demonstrate that the presence of a poly(A) tail on a target mRNA can increase the magnitude of let-7-mediated translational repression. These results mirror our previous observations with the miCXCR4 reporter system [19], but they are counter to previous observations that the poly(A) tail did not contribute to repression of R-luc-3xb mRNA by let-7 [18]. Normalization to the parental RL mRNA instead of R-luc-mut (Fig. S5 and Supporting Information S1) may have masked a poly(A) tail contribution to let-7-mediated repression in previous work [18].

A block to let-7-mediated deadenylation impairs translational repression

Finally, we devised a strategy to specifically interfere with poly(A) tail removal, which involved sterically blocking the 3′ end of the target mRNA’s poly(A) tail. To achieve this, we prepared capped R-Luc (3xb or mut) mRNAs carrying a poly(A) tail of defined length (cap& Fig. 5A). These mRNA were transcribed in vitro from plasmids that were modified to include an A₉₂ segment downstream of the let-7 target sites to template the poly(A) tail (see Materials & Methods for plasmid details). The plasmid templates were linearised just downstream of this segment so that the A₉₂ tail of the resulting mRNAs was followed by an 8 nt heteropolymeric 3′ tag. We first ascertained that this A₉₂ tail still strongly stimulated translation, by comparison of cap&₉₂ with cap only versions of the R-luc-mut mRNA after transfection into HeLa cells (data not shown). We also found that appending the A₉₂ tail and 8 nt 3′ tag instead of a longer poly(A) tail to the R-luc reporter mRNAs by itself did not affect on let-7-mediated repression, which was still strong (~10.5 fold repression, measured 16 hours after mRNA transfection, Fig. 5C). This suggested that there is little/no variance in the mechanism of mRNA action in the presence of the 8 nt tag 3′ of the poly(A) tail.

To block access of deadenylases to the mRNA 3′ end, we co-transfected HeLa cells with R-luc-(3xb or mut)-cap&₉₂ mRNA and the ‘tail-blocker’ (tb) LNA complementary to the 3′ tag or an unrelated control LNA (ns). We found that expression of R-protein from the R-luc-mut mRNA tended to be mildly elevated in the presence of tb LNA compared to transfection of non-specific control LNA or mock transfection (Fig. 5B), consistent with a block of default deadenylation of this mRNA. Importantly, we observed that the tb LNA specifically reduced let-7-mediated repression to ~6-fold (Fig. 5C). tb LNA co-transfection had no appreciable effect on either physical (data not shown) or functional stability (Fig. 3D) of
**Figure 3.** *let-7*-mediated repression is relieved by depletion of RCK/p54 or TNRC6A despite target mRNA deadenylation. HeLa cells were transfected with siRNAs targeting *GW182*, *RCK*, the non-specific miCXCR4 control (ns), or mock transfected (none) as indicated. (A) Residual mRNA levels were measured by qPCR between 24 and 48 hours and normalised against *RPL13a*. Bars represent the average expression of the relevant mRNA in three independent experiments with standard error. mRNA expression in cells transfected with ns was set at 1 (hence do not carry an associated error). (B) Extracts from cells harvested 24 or 48 hours after RCK or control siRNA transfection were analysed by western blotting and infrared fluorescent imaging using antibodies against RCK and tubulin. RCK protein level (normalised to tubulin loading control) was reduced to 25% (24 hours) and 11% (48 hours). * Cross-reacting non-specific band. (C, D) After 24 hours of knockdown, cells were transfected with *let-7* reporter plasmids. R-luc activity (C) and mRNA levels (D) were measured 24 hours later and repression calculated as in Fig. 1. Derepression achieved by siRNA knockdown was calculated as 1 - (repression in siRNA treated cells/repression in "none" cells) and normalised to derepression by *let-7* anti-miR seen in parallel transfections (set to 100% derepression, Fig. 1B. Bars represent averages of 4–10 (a) or 4–5 (b) independent experiments with standard error. (E) HeLa cells were transfected as above and total RNA purified 6 hours after plasmid transfection for LM-PAT assay of the R-luc and *GAPDH* mRNAs. Data representative of 3–4 independent experiments are shown. (F) After 30 hours of knockdown, cells were transfected with *in vitro* transcribed capped R-luc-mut mRNA with or without a poly(A) tail (expression of R-luc-mut with poly(A) tail in "none" set to 1.0 for each experimental set prior to averaging across experiments). Averaged results from 3–7 independent experiments are shown with standard error.

doi:10.1371/journal.pone.0006783.g003
R-Luc/cap&A62 mRNA, indicating that the tb LNA impaired repression at the level of translation. We were unable to measure the tail length dynamics of the R-luc cap&A62 mRNA in transfected cells as the 3'9 tag interfered with the LM-PAT assay (data not shown). To demonstrate that the tb LNA prevented reporter mRNA deadenylation as intended, we incubated our R-luc mRNAs in HeLa cell extracts [63,64], which we found capable of enacting let-7-mediated deadenylation. Using this system as a surrogate, we confirmed that the tb LNA protected the 3' end (Fig. S6).

Altogether, these results show that removal of the poly(A) tail is not essential for miRNA-mediated translational repression. Nevertheless, they also indicate that deadenylation can augment miRNA-mediated translational repression in mammalian cells.

Discussion

We show here using a reporter system in HeLa cells that let-7 triggers target mRNA deadenylation, which is evident before measurable translational repression and mRNA destabilisation. The presence of a poly(A) tail on target mRNA enhances repression, while blocking tail removal by deadenylation reduces the magnitude of translational repression. Nevertheless, let-7-mediated repression can be attenuated despite ongoing deadenylation as observed in cells depleted of RCK or TNRC6A. Thus deadenylation is not essential for, but can quantitatively contribute to, let-7-mediated translational repression. Our findings are also consistent with a role of deadenylation in miRNA-mediated mRNA decay. In this way, we concur with and extend similar conclusions reached in other systems as referred to throughout this paper. A distinguishing feature of the present study is its detailed description of the relationship between miRNA-mediated deadenylation and translational repression in mammalian cells.

mRNA polyadenylation control is a versatile means to regulate gene expression [47,65] and mRNA deadenylation is emerging as a widespread feature of miRNA action. In zebrafish embryos, miR-430 facilitates the clearance of hundreds of maternal mRNA polyadenylation.
Figure 5. Blocking mRNA deadenylation impairs let-7-mediated translational repression. (A) Schematic of the R-luc 3x6 and mutant 3x6/cap&A mRNA indicating the site of 'tail blocker' (tb) binding. (B, C) HeLa cells were co-transfected with R-luc reporter mRNAs and LNA as indicated (as for Fig. 4D,E). Cells were harvested at different times to measure R-luc protein expression, which was normalized to F-luc. Average results from 4-7 independent triplicate experiments are shown with standard error. (D) Multiple cell aliquots were co-transfected with R-luc reporter mRNAs and LNA as indicated (as for Fig. 4D). Cells were harvested at different times to measure R-luc protein expression, which was normalized to F-luc. Two time series of this kind (with duplicate measurements) were each scaled to total level of recovered R-luc protein and then averaged. Error bars represent data range.

PLOS ONE | www.plosone.org 8 August 2009 | Volume 4 | Issue 8 | e6783

miRNA & mRNA Deadenylation

decaying in D. melanogaster [41]. Microarray analyses demonstrated that 60% of mRNA stabilised in the absence of Ago 1 were also increased in cells depleted of two different CCR4:NOT subunits [42]. We show here that miRNA-mediated mRNA deadenylation is readily observable with both reporters and endogenous mRNAs in mammalian cells.

Deadenylation is the first step in canonical mRNA turnover [66] and it thus contributes to the decay-promoting activity of miRNA. Its relationship with miRNA-mediated translational repression has been less clear. Deadenylation could arise as a consequence of translational repression by a miRNA. This is a plausible hypothesis for which there is precedent [67]. However, it has been shown in zebrafish, mammalian and D. melanogaster cells that blocking translation initiation on reporter mRNAs by interfering with either 40S recruitment [39,42], 5’ UTR scanning [40], or start codon recognition [38] does not prevent miRNA-mediated deadenylation. We add our observation that miRNA-mediated deadenylation is evident prior to the onset of measurable translational repression. Taken together, it is thus apparent across different systems that miRNAs can stimulate deadenylation without a specific requirement for mRNA translation or a prior translational repression step.

Alternatively, deadenylation may exclusively stimulate mRNA decay. This has been addressed in mammalian and D. melanogaster cells using reporter constructs designed to give rise to mRNAs either carrying the 3’ end stem-loop of histone mRNA instead of a tail, or lacking a tail due to the presence of a self-cleaving ribozyme in the 3’ UTR [40,42,46]. These mRNAs could still be silenced, although at times not as strongly as their polyadenylated counterparts. The common observation is thus that miRNAs can repress translation without a requirement for mRNA deadenylation. It does, however, not necessarily follow that deadenylation has no role in translational repression, as shown by our present findings and previous reports that translational repression is enhanced by the presence of a poly(A) tail [19,20,25,28]. Most importantly, we show here that while blocking let-7-mediated deadenylation does not completely abolish let-7-mediated translational repression, it nevertheless clearly attenuated it. This is a direct demonstration in living cells that mRNA deadenylation can specifically contribute to translational repression by a miRNA.

Taken together with the extant literature, our data thus favours a model whereby mRNA deadenylation is a proximal mRNA action that not only promotes mRNA decay but also attenuates the stimulatory role of the poly(A) tail in mRNA translation, by generating an oligo-adenylated form of the mRNA that associates with cytoplasmic PABP. Indeed, mammalian miRNA target mRNAs are enriched in Ago and GW182 immunoprecipitations, while they are underrepresented in complexes containing PABPC4 [12]. The oligo-adenylated mRNA is further silenced by co-repressors, which most plausibly interfere with cap/eIF4E function [18,19,20,23,24,25,26,28]. Indeed, mammalian miRNA target mRNAs are enriched in Ago and GW182 immunoprecipitations, while they are underrepresented in complexes containing PABPC4 [12]. The oligo-adenylated mRNA is further silenced by co-repressors, which most plausibly interfere with cap/eIF4E function [18,19,20,23,24,25,26,28]. Most importantly, we show here that while blocking let-7-mediated deadenylation does not completely abolish let-7-mediated translational repression, it nevertheless clearly attenuated it. This is a direct demonstration in living cells that mRNA deadenylation can specifically contribute to translational repression by a miRNA.

at the onset of zygotic transcription [38], and reporters encoding 3’ UTRs from several of these mRNAs were shown to be deadenylated in a miR-430-dependent manner [38,39]. miR-125b or let-7 were shown to hasten deadenylation and decay of responsive reporters in mammalian cells [40]. In vitro systems to study miRNA-mediated translational repression of mammalian or D. melanogaster origin also feature miRNA-mediated deadenylation [25,28]. Degradation of miRNA-sensitive 3’ UTR reporters was shown to involve the CCR4:NOT deadenylase and DCP1:DCP2...
given cellular environment. With these provisos, the model explains most reports assessing the role of the poly(A) tail in miRNA-mediated repression.

The above scenario clearly does not accommodate post-initiation effects by miRNAs, and several reports have further presented experimental evidence or theoretical arguments to link miRNA-mediated repression to late sub-steps of translation initiation [22,71,72,73]. More work will have to be done to address the possibility that miRNAs affect translation at more than one step. The poly(A) tail also stimulates the 60S subunit joining step during late initiation and it is linked to translation termination through an interaction between PABP and eRF3 [44], providing ways to rationalise a role for deadenylation in several alternative models of translational repression by miRNAs.

**Materials and Methods**

**DNA constructs, siRNAs and LNA oligonucleotides.**

**DNA constructs.** The plasmids pRL, pRL-3xbulge and pRL-3xbulgemut, which encode Renilla luciferase driven from a CMV promoter, were gifts from W. Fillipowicz (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland) [18]. The three let-7 binding sites in R-luc-3xb are engineered ~10 nt downstream of the stop codon. Plasmid transfection into cells results in production of R-luc mRNAs with a 3′UTR ~260 nt in length and the poly(A) tail added at an SV40 late poly(A) signal ~160 nt from the last let-7 binding site. R-luc-3xb/mut mRNAs transcribed from these plasmids in vitro differ slightly in that transcription begins at the T7 promoter and the poly(A) tail is added at the HpaI site 7 nt preceding the SV40 poly(A) signal cleavage site. The Firefly luciferase pGDL3 plasmid was obtained from Promega. The pRL-A62 constructs (Fig. 5) were engineered from pRL-3xb and pRL-mut backbones. Briefly, this involved inserting a Not I fragment from pRL-WT [23] (containing six mi-2 binding sites and a stretch of 62 adenosines) into the Not I site of the pRL-3xb and pRL-mut constructs (within the R-luc 3′UTR). The six mi-2 binding sites were then removed by digestion with SacII and re-ligation of the remaining backbone, creating the pRL-A62 constructs. Linearisation of this pRL-pA62 construct with EcoICR1 enzyme (Promega) creates an eight nucleotide overhang after the poly(A) tail stretch. The in vitro transcribed transcripts of RL-3xb/mut-cap&A62 mRNA encode a 3′UTR of ~107 nt, with the encoded poly(A) starting 20 nt downstream of the last-7 mRNA binding site.

**Locked nucleic acid (LNA) oligonucleotides.** LNA modified anti-miR oligonucleotides were obtained from Exiqon (MA, USA) with the following sequences: hsa-miR-10-7a, AACTATAC-AACCTACTACCTCA, hsa-miR-499, TTAAACATCACTG-USA) with the following sequences: hsa-let-7a, AACTATAC-3. miCR2 was used in these experiments as an miRNA & mRNA Deadenylation template for miRNA-mediated repression. The above scenario clearly does not accommodate post-initiation effects by miRNAs, and several reports have further presented experimental evidence or theoretical arguments to link miRNA-mediated repression to late sub-steps of translation initiation [22,71,72,73]. More work will have to be done to address the possibility that miRNAs affect translation at more than one step. The poly(A) tail also stimulates the 60S subunit joining step during late initiation and it is linked to translation termination through an interaction between PABP and eRF3 [44], providing ways to rationalise a role for deadenylation in several alternative models of translational repression by miRNAs.

**mRNA transfections.** For mRNA transfections, cells were seeded in a 24-well plate and transfections were performed in triplicate at ~60–70% confluency by adding a preincubated (30min, 25°C) transfection solution (200 μl) and 300 μl of OptiMEM I (Invitrogen) to each well. The 200 μl of transfection solution (in Opti-MEM I) contained 1 μl Lipofectamine 2000 (Invitrogen), 10 or 20 ng Renilla luciferase-encoding (R-luc) mRNA and 80 ng pGL3 (F-luc control). Cells were harvested 16 hours after transfection unless stated otherwise. For anti-miRNA transfections (Fig. 1), 200 μl of transfection solution also contained 5 pmoles of LNA. For the tail blocking experiments (Fig. 5), 1.6 pmoles of tail blocking (tb) or non-specific (ns) LNA was added to the transfection solution. For depletion studies (Fig. 3), siRNA was transfected into cells at ~35–50% confluency 30 hours prior to mRNA transfection. To transfect one well, 100 μl of transfection solution (in Opti-Mem I) contained 10 pmole siRNA and 1 μl RNAmax Lipofectamine (Invitrogen) and was transfected into cells according to the manufacturer’s instructions. 4–8 hours after transfection the medium was replaced with normal growth medium. Subsequent mRNA transfections were as described above except the 200 μl of mRNA transfection solution was added to 300 μl of normal growth medium (not Opti-Mem I). When using synthetic miRNA (Fig. S4) the transfection solution additionally contained 1 pmole of duplex miRNA.

**Plasmid transfections.** Plasmid transfections were performed similarly to mRNA, with the 200 μl transfection solution containing 30 ng of R-luc encoding plasmid and 600 ng of pGL3 F-luc. Medium was replaced ~8 hours after transfection and cells were harvested at times specified. Additionally, in the time-course described in Fig. 2 and Fig. S2, cells were trypsinised and diluted two-fold to maintain an actively growing culture. For anti-miRNA transfections (Fig. 1B, D, Fig. 4), 200 μl of plasmid transfection solution also contained 5 pmoles of LNA. For the depletion studies (Fig. 3), siRNA was transfected into cells 24 hours prior to plasmid transfections as described above for mRNA transfections.

**P19 cell differentiation.** For Fig. 1E, P19 cells were induced to differentiate in 500 nM retinoic acid (RA) on 15 cm2 bacterial grade plastic in the absence of RA on Day 4. Cells were seeded and P19 cells were maintained in DMEM with 5% FCS, supplemented with glutamine and penicillin/streptomycin, as detailed in [19,48]. Mouse embryonal carcinoma P19 cells were maintained in the same media and induced to differentiate as described [76]. Growth conditions for SH-SY5Y cells (Fig. S1) were as previously reported [77].

**Cell culture, transfections and Dual luciferase assays.** HeLa cells were maintained in DMEM with 5% FCS, supplemented with glutamine and penicillin/streptomycin, as detailed in [19,48]. Mouse embryonal carcinoma P19 cells were maintained in the same media and induced to differentiate as described [76]. Growth conditions for SH-SY5Y cells (Fig. S1) were as previously reported [77].

**Plasmid transfections.** Plasmid transfections were performed similarly to mRNA, with the 200 μl transfection solution containing 30 ng of R-luc encoding plasmid and 600 ng of pGL3 F-luc. Medium was replaced ~8 hours after transfection and cells were harvested at times specified. Additionally, in the time-course described in Fig. 2 and Fig. S2, cells were trypsinised and diluted two-fold to maintain an actively growing culture. For anti-miRNA transfections (Fig. 1B, D, Fig. 4), 200 μl of plasmid transfection solution also contained 5 pmoles of LNA. For the depletion studies (Fig. 3), siRNA was transfected into cells 24 hours prior to plasmid transfections as described above for mRNA transfections.

**Luciferase Reporter assays.** These were performed using the Dual-Luciferase Reporter Assay system (Promega) in a FLUOstar Optima plate reader (BMG Labtech) as described [19].

**Quantitative Western blotting.** SDS-PAGE gels were transferred to Immobilon-FL membrane (Millipore). Anti-RCK (MBL International) and α-Tubulin antibodies (Santa Cruz) were
used at 1:1000 working dilution. Fluorescent secondary antibodies were anti-rabbit/alexa-750 and anti-mouse/alexa-600 conjugated antibodies (Invitrogen). Blot hybridisation and detection was performed using the Odyssey Infrared Imaging System and buffers according to the manufacturer’s instructions (LI-COR Biosciences).

**RNA analyses**

**Purification of total RNA from cells in culture.** This was done using Trizol (Invitrogen) according to the manufacturer’s instructions.

**Quantitative reverse transcriptase PCR.** For analysis of reporter mRNA levels (Figs. 1B, 2, 3), HeLa cells were transfected in 12-well plates by scaling up the plasmid transfection protocol detailed above. Before RNA purification, cells were trypsinised and washed in PBS to remove unincorporated transfection mixtures. Purified total RNA was then treated with Turbo DNase (Ambion) as per the manufacturer’s instructions. 300-1000 ng of RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) using a random hexamer primer, as per manufacturer’s instructions, while a no-RT control was set up in parallel to assess the efficiency of the DNase treatment. mRNA levels were assessed by Quantitative (q)PCR on a Timecourse of protein and mRNA expression in transiently transfected HeLa cells. HeLa cells were cotransfected with either pRL-3xbulge or pRL-3xbulgemut plasmid [8] and pGL3 (F-luc control). Protein and RNA was harvested from cells 1–48 hours after plasmid transfection. Cells were trypsinised and diluted two-fold at 24 hours to maintain actively growing cultures. (A) Raw R-luc activity (prior to normalisation). Points represent average of triplicates with standard error. (B) R-luc mRNA levels were measured by qPCR and normalised to total input RNA. Points represent average of triplicates with standard error. These values were further used to calculate repression as depicted in Fig. 2A. To this end, R-luc levels (mRNA or protein) at each time point were further normalised to the F-luc transfection control. No repression was calculated for the one-hour time point as values were near background. Found at: doi:10.1371/journal.pone.0006783.s002 (1.07 MB EPS)

**Figure S2** Timecourse of protein and mRNA expression in transiently transfected HeLa cells. HeLa cells were cotransfected with either pRL-3xbulge or pRL-3xbulgemut plasmid [8] and pGL3 (F-luc control). Protein and RNA was harvested from cells 1–48 hours after plasmid transfection. Cells were trypsinised and diluted two-fold at 24 hours to maintain actively growing cultures. (A) Raw R-luc activity (prior to normalisation). Points represent average of triplicates with standard error. (B) R-luc mRNA levels were measured by qPCR and normalised to total input RNA. Points represent average of triplicates with standard error. These values were further used to calculate repression as depicted in Fig. 2A. To this end, R-luc levels (mRNA or protein) at each time point were further normalised to the F-luc transfection control. No repression was calculated for the one-hour time point as values were near background. Found at: doi:10.1371/journal.pone.0006783.s002 (1.07 MB EPS)

**Figure S3** Microfluidic assessment of in vitro transcribed miRNAs. (A) Schematic of the variant R-luc miRNAs. (B) Variant R-luc-3xb and mut mRNA, as well as parental R-luc mRNA, were transcribed in vitro and analysed by microfluidics. Found at: doi:10.1371/journal.pone.0006783.s004 (0.82 MB EPS)

**Figure S4** The mRNA poly(A) tail contributes to translational repression by synthetic let-7a. (A) Endogenous expression of let-7 in HeLa cells and P19 cells, either undifferentiated (−) or differentiated by retinoic acid treatment (RA), was measured by northern blotting (100 μg total RNA loaded per lane). (B, C) Undifferentiated P19 cells were co-transfected with R-luc mRNA (3xb or mut), pGL3 (F-luc control) and synthetic let-7 or control miRNA followed by incubation for 16 hours. (B) R-luc activity from the R-luc-mut miRNAs transfected into P19 cells (normalised to F-luc reference; expression from the cap&&tail mRNA is set to 1.0). (C) Repression by synthetic let-7a in P19 cells was calculated by dividing the normalized R-luc activity from cells co-transfected with the miCXCR4 control by the normalized R-luc activity from cells co-transfected with let-7a (filled bars: R-luc-3xb mRNA, open...
bars: R-luc-mut mRNA). Averaged results from 4-7 independent triplicate repeat experiments are shown with standard error in B and C.

Found at: doi:10.1371/journal.pone.0006783.s005 (1.71 MB EPS)

Figure S5 Normalisation to R-luc-RL mRNA can mask the poly(A) tail dependence of miRNA-mediated repression. (A,B) HeLa cells were co-transfected with R-luc mRNA (3xb or RL) and pGL3 (F-luc control) and incubated for 16 hours. (A) R-luc activity from the R-luc-RL mRNAs transfected into HeLa cells (normalised to F-luc reference; expression from the cap&tail mRNA is set to 1.0). (B) Repression by endogenous let-7 in HeLa cells. Repression was calculated as for Fig. 1. Averaged results from 5 independent triplicate repeat experiments are shown with standard error.

Found at: doi:10.1371/journal.pone.0006783.s006 (0.49 MB EPS)

Figure S6 The tb LNA blocks let-7-mediated mRNA deadenylation in vitro. (A) Schematic of the R-luc 3xb and mutant cap&62 mRNAs indicating the site for oligonucleotide-mediated RNase H cleavage. (B) R-luc 3xb or mutant cap&62 mRNAs were incubated in cell-free translation reactions based on HeLa extract for 30–180 min as indicated. Recovered RNA was subjected to RH-R-Luc oligonucleotide-mediated cleavage by RNase H and northern blot analysis as in Fig. 1C. Labels to the left of the panel mark the position of the 3′ UTR fragments carrying different lengths of poly(A) tail. (C) As in B, except that reactions additionally contained anti-miR LNAs as indicated. Reactions were incubated for 120 min. Control cleavage reactions with input R-luc 3xb cap&62 mRNA in the presence or absence of oligo(dT) generate 3′ UTR fragments with or without intact poly(A) tail, shown on the right. (D) As in C, except that R-luc 3xb cap&62 mRNA was annealed to specific (tb) or control (ns) LNA prior to incubation. Control cleavage reactions are shown on the left; probing for U6 snRNA as a loading control is shown in the lower panel.

Found at: doi:10.1371/journal.pone.0006783.s007 (16.18 MB EPS)

Table S1 Gene specific primer sequences for quantitative PCR and LM-PAT assays.

Found at: doi:10.1371/journal.pone.0006783.s008 (0.04 MB DOC)

Acknowledgments

We thank W. Fillipowicz for the pRL-3xb bulge and -mut constructs and N. Schourock for a sample of SH-SY5Y cells.

Author Contributions

Conceived and designed the experiments: DM TP. Performed the experiments: THB DTH JC. Analyzed the data: THB DTH JC. Contributed reagents/materials/analysis tools: RT MWH. Wrote the paper: MWH TP.

References

1. Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. Dev Cell 11: 441–450.

2. Chang TC, Mendell JT (2007) microRNAs in vertebrate physiology and human disease. Annu Rev Genomics Hum Genet 8: 215–239.

3. Peters L, Meister G (2007) Argonaute proteins: mediators of RNA silencing. Mol Cell 26: 611–623.

4. Gu S, Jin L, Zhang F, Sarnow P, Kay MA (2009) Biological basis for restriction of microRNA targets to the 3′ untranslated region in mammalian mRNAs. Nat Struct Mol Biol 16: 144–150.

5. Enalalo A, Huentzinger E, Izaurrealde E (2008) Getting to the root of mRNA-mediated gene silencing. Cell 132: 9–14.

6. Fillipozcow, B, Bhattacharrya SN, Sonenberg N (2008) Mechanisms of post-transcriptional repression by microRNAs: are the answers in sight? Nat Rev Genet 9: 102–114.

7. Wu L, Belasco JG (2008) Let me count the ways: mechanisms of gene regulation mediated by microRNAs. Mol Cell 29: 1–7.

8. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006) Control of translation initiation level of translation requires GW182 proteins. EMBO J 28: 213–222.

9. Sekelsky, J, Schwannacker B, Thielecker F, Fang M, Inman R, et al. (2000) Widespread changes in protein synthesis induced by microRNAs. Nature 453: 58–63.

10. Back D, Villeden J, Shin C, Camargo FO, Gygi S, et al. (2008) The impact of microRNAs on protein output. Nature 453: 64–71.

11. Pillai RS, Arna CG, Filippowicz W (2004) Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA 10: 1518–1523.

12. Landthaler M, Gardatzis D, Rother M, Chen PY, Soll SJ, et al. (2008) The impact of microRNAs on protein output. Nature 453: 64–71.

19. Humphreys DT, Westman BJ, Martin DL, Preiss T (2005) MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci U S A 102: 16961–16966.

20. Wang B, Love TM, Call ME, Doench JG, Novina CD (2006) Recapitulation of Short RNA-Directed Translational Gene Silencing In Vivo. Mol Cell 22: 553–560.

21. Bhattacharyya SN, Habermacher R, Martine U, Closs EJ, Fillipowicz W (2006) Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 125: 1111–1124.

22. Chengrimada TP, Finn KJ, Ji X, Bailand D, Gregory RI, et al. (2007) MicroRNA silencing through RISC recruitment of eIF6. Nature 447: 823–828.

23. Therian M, Hentze MW (2007) Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature 447: 875–878.

24. Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT, et al. (2007) An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell 129: 1141–1151.

25. Wakiyama M, Takimoto K, Ohta O, Yokoyama S (2007) Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev 21: 1857–1862.

26. Mathonnet G, Fabian MR, Svintik YV, Parsyan A, Huck L, et al. (2007) MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science 317: 1764–1767.

27. Hong WW, Cannell IG, de Moor CH, Hill K, Garside PG, et al. (2008) The mechanism of microRNA-mediated translation repression is determined by the promoter of the target gene. Proc Natl Acad Sci U S A 105: 8866–8871.

28. Jovean K, Kawaizuma T, Tomari Y (2009) Drosophila Argonaute1 and Argonaute2 Employ Distinct Mechanisms for Translational Repression. Mol Cell.

29. Olsen PH, Ambros V (1999) The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 216: 671–680.

30. Seggerson K, Tang L, Moss EG (2002) Two genetic circuits repress the two genetic circuits repress the translation initiation. Dev Biol 216: 213–222.

31. Chekliarev M, Fillipowicz W, Parker R (2009) Multiple independent domains of dGW182 function in mRNA-mediated translational repression in Drosophila. RNA 15: 794–803.

32. Lazzarette D, Tournier I, Izaurrealde E (2009) The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. RNA 15: 1059–1066.

33. Enalalo A, Helms S, Fritzsche C, Fawer M, Izaurrealde E (2009) The C-terminal silencing domain in GW182 is essential for miRNA function. RNA 15: 1067–1077.

34. Pillai RS, Bhattacharyya SN, Arna CG, Zoller T, Cougot N, et al. (2005) Inhibition of Translational Initiation by let-7 MicroRNA in Human Cells. Science 309: 1573–1576.
35. Nilsen TW (2007) Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet 23: 243–249.
36. Standart N, Jackson RJ (2007) MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev 21: 1975–1982.
37. Chekulaeva M, Filipowicz W (2009) Mechanisms of microRNA-mediated post-transcriptional regulation in animal cells. Curr Opin Cell Biol 21: 452–460.
38. Giraldes AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, et al. (2006) Zebrafish mir-430 promotes deadenylation and clearance of maternal mRNAs. Science 312: 75–79.
39. Mishima Y, Giraldes AJ, Takeda Y, Fujiwara T, Sakamoto H, et al. (2006) Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. Curr Biol 16: 2133–2142.
40. Wu L, Fan J, Belasco JG (2006) MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103: 4034–4039.
41. Belousov I, Rehwinkel J, Doerks T, Stark A, Bork P, et al. (2006) miRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev 20: 1885–1898.
42. Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, Fauser M, et al. (2009) Deadenylation is a widespread effect of miRNA regulation. RNA 15: 21–32.
43. Jacobson A (1996) Poly(A) metabolism and translation: the closed-loop model. In: Hershey JWB, Mathews MB, Sonenberg N, eds (1996) Translational control. Cold Spring Harbor N.Y.: Cold Spring Harbor Laboratory Press. pp 451–480.
44. Preiss T, Hentze MW (2003) Starting the protein synthesis machine: eukaryotic translation initiation. Bioessays 25: 1201–1211.
45. Amrani N, Ghosh S, Mangus DA, Jacobson A (2008) Translation factors promote the formation of two states of the closed-loop mRNP. Nature 453: 1276–1280.
46. Eulalio A, Huntzinger E, Izaurralde E (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol 15: 346–353.
47. Beilharz TH, Preiss T (2007) Widespread use of poly(A) tail length control to accentuate expression of the yeast transcriptome. RNA 13: 982–997.
48. Clancy JL, Nousch M, Humphreys DT, Westman BJ, Beilharz TH, et al. (2007) Methods to analyze microRNA-mediated control of mRNA translation. Methods Enzymol 431: 83–111.
49. Lee YS, Dutta A (2007) The tumor suppressor microRNA let-7 represses the HMGAA2 oncogene. Genes Dev 21: 1025–1030.
50. Mayr C, Hemann MT, Bartel DP (2007) Disrupting the pairing between let-7 and Hmgaa2 enhances oncogenic transformation. Science 315: 1576–1579.
51. Sempere LF, Freemantle S, Pita-Rosse I, Moss E, Dmitrovsky E, et al. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol 5: R13.
52. Karginov FV, Conaco C, Xuan Z, Schmidt BH, Parker JS, et al. (2007) A biochemical approach to identifying microRNA targets. Proc Natl Acad Sci U S A 104: 19291–19296.
53. Beitzinger M, Peters L, Zhu JY, Kremmer E, Meister G (2007) Identification of human microRNA targets from isolated argonaute protein complexes. RNA Biol 4: 76–84.
54. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129: 1401–1414.
55. Chu CY, Rana TM (2006) Translation repression in human cells by MicroRNA-Induced Gene Silencing Requires RCK/p54. PLoS Biol 4: e210.
56. Meister G, Landthaler M, Peters L, Chen PY, Uzlaub H, et al. (2005) Identification of novel argonaute-associated proteins. Curr Biol 15: 2149–2155.
57. Jakymiw A, Lian S, Estaysthov T, Li S, Satoh M, et al. (2005) Disruption of GW bodies impairs mammalian RNA interference. Nat Cell Biol 7: 1167–1174.
58. Liu J, Rivas FV, Wohлечegel J, Yates JR 3rd, Parker R, et al. (2005) A role for the P-body component GW182 in microRNA function. Nat Cell Biol 7: 1161–1166.
59. Till S, Lejeune E, Ther mann R, Bortfeld M, Hothorn M, et al. (2007) A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. Nat Struct Mol Biol 14: 897–903.
60. Eulalio A, Rehwinkel J, Stricker M, Huntzinger E, Yang MF, et al. (2007) Target-specific requirements for enhancers of decapping in microRNA-mediated gene silencing. Genes Dev 21: 2550–2570.
61. Barreau C, Dutertre S, Paillard L, Osborne HB (2006) Liposome-mediated RNA transfection should be used with caution. RNA 12: 1790–1793.
62. Galle R (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev 5: 2106–2116.
63. Bergamini G, Preiss T, Hentze MW (2006) Picoauravir IRE3es and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system. RNA 6: 1781–1790.
64. Westman B, Beeren L, Gradzien E, Stepinsz J, Worch R, et al. (2005) The antiviral drug ribavirin does not mimic the 7-methylguanosine moiety of the mRNA cap structure in vitro. RNA 11: 1505–1513.
65. Goldstrohm AC, Wickens M (2008) Multifunctional deadenylase complexes diversify mRNA control. Nat Rev Mol Cell Biol 9: 337–344.
66. Beelman C, P R (1995) Degradation of mRNA in Eukaryotes. Cell 81: 179–183.
67. Muckenthaler M, Gunkel N, Stripecke R, Hentze MW (1997) Regulated poly(A) tail shortening in somatic cells mediated by cap-proximal translational repressor proteins and ribosome association. RNA 3: 983–995.
68. Kuhn U, Wahle E (2004) Structure and function of poly(A) binding proteins. Biochim Biophys Acta 1678: 67–84.
69. Mangus DA, Evans MC, Jacobson A (2003) Poly(A)-binding proteins: multifunctional scaffolds for the post-transcriptional control of gene expression. Genome Biol 4: 223.
70. Yoshida M, Yoshida K, Koulou G, Lim NS, De Crescenzo G, et al. (2006) Poly(A) binding protein (PABP) homoeostasis is mediated by the stability of its inhibitor, Paip2. EMBO J 25: 1935–1944.
71. Wang B, Yanez A, Novina CD (2003) MicroRNA-expressed mRNAs contain 48b but not 60s components. Proc Natl Acad Sci U S A 105: 5343–5348.
72. Ding XY, Slack FJ, Grosshans H (2008) The let-7 microRNA interface extensively with the translation machinery to regulate cell differentiation. Cell Cycle 7: 3003–3009.
73. Nissan T, Parker R (2008) Computational analysis of miRNA-mediated repression of translation: implications for models of translation initiation inhibition. RNA 14: 1480–1491.
74. Doench JG, Petersen CP, Sharp PA (2003) siRNAs can function as miRNAs. Genes Dev 17: 409–414.
75. Doench JG, Sharp PA (2004) Specificity of microRNA target selection in translational repression. Genes Dev 18: 504–511.
76. Gill RM, Slack R, Kress M, Hamed PA (1998) Regulation of expression and activity of distinct pRB, E2F, D-type cyclins, and CK1 family members during terminal differentiation of P19 cells. Exp Cell Res 244: 157–170.
77. Ferrari A, Hoerndli F, Bacchi T, Nitsch RM, Gotz J (2003) beta-Amyloid induces paired helical filament-like tau filaments in tissue culture. J Biol Chem 278: 40162–40168.
78. Beilharz TH, Preiss T (2009) Transcriptome-wide measurement of mRNA polyadenylation state. Methods 48: 294–300.