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Repression of *C. elegans* microRNA targets at the initiation level of translation requires GW182 proteins

Xavier C Ding and Helge Großhans*

Friedrich Miescher Institute for Biomedical Research (FMI), Basel, Switzerland

MicroRNAs (miRNAs) repress target genes through a poorly defined antisense mechanism. Cell-free and cell-based assays have supported the idea that miRNAs repress their target mRNAs by blocking initiation of translation, whereas studies in animal models argued against this possibility. We examined endogenous targets of the *let-7* miRNA, an important regulator of stem cell fates. We report that *let-7* represses translation initiation in *Caenorhabditis elegans*, demonstrating this mode of action for the first time in an organism. Unexpectedly, although the *lin-4* miRNA was previously reported to repress its targets at a step downstream of translation initiation, we also observe repression of translation initiation for this miRNA. This repressive mechanism, which frequently but not always coincides with transcript degradation, requires the GW182 proteins AIN-1 and AIN-2, and acts on several mRNAs targeted by different miRNAs. Our analysis of an expanded set of endogenous miRNA targets therefore indicates widespread repression of translation initiation under physiological conditions and establishes *C. elegans* as a genetic system for dissection of the underlying mechanisms.

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Introduction

MicroRNAs (miRNAs) are small, untranslated RNAs involved in numerous developmental pathways in plants and animals (reviewed in Bushati and Cohen, 2007). They regulate a large fraction of cellular mRNAs by binding to complementary sequences in their target mRNAs ('cognate mRNAs'), but the mechanisms involved in subsequent repression of the mRNA are less clear (reviewed in Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). In the best understood example, prevalent in plants, miRNAs function as small interfering (si)RNAs and induce mRNA cleavage through the RNA-induced silencing complex (RISC) when binding to perfectly complementary sites in their target mRNAs (Jones-Rhoades *et al*, 2006). In animals, this appears to be the exception, as most animal miRNAs are only partially complementary to their targets (Bushati and Cohen, 2007), thus precluding RISC-mediated cleavage. Early work on the *Caenorhabditis elegans lin-4* miRNA established, instead, the paradigm that miRNAs functioned by translationally repressing their targets at a step downstream of translation initiation, without substantially affecting transcript levels (Olsen and Ambros, 1999; Seggerson *et al*, 2002). By contrast, recent studies aimed at recapitulating miRNA function in cell-free systems concluded that miRNAs inhibit target mRNA translation at the initiation step (Wang *et al*, 2006; Mathonnet *et al*, 2007; Thermann and Hentze, 2007; Wakiyama *et al*, 2007). Inhibition of translation initiation, as evidenced by the hallmark shift of target mRNAs from heavy to light polysomal or monosomal fractions of sucrose density gradients in response to the miRNA, has also been observed in a number of cell-based studies. However, such studies also identified additional and sometimes conflicting miRNA modes of action (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). These mechanisms include inhibition of target mRNA translation after initiation, target mRNA degradation in a non-endonucleolytic manner, which may or may not result from deadenylation, and co-translational protein degradation. Target mRNA degradation has also been observed for some miRNA targets *in vivo*, in *C. elegans* and zebrafish (Bagga *et al*, 2005; Giraldez *et al*, 2006).

Only a single study has so far demonstrated regulation of an endogenous mRNA, CAT1, by its cognate miR-122 miRNA at the level of translation initiation (Bhattacharyya *et al*, 2006). The other studies that examined endogenous miRNA targets instead provided evidence against repression of translation initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002; Kong *et al*, 2008), and this includes the only two studies that have tested this mechanism in an animal model, under physiological conditions (Olsen and Ambros, 1999; Seggerson *et al*, 2002). It is currently unclear whether this divergence of results denotes specific mechanisms operating for individual miRNAs and/or targets. Alternatively, the transfected miRNA target reporters that were used in the bulk of studies showing repression of translation initiation by miRNAs might be particularly conducive to this mode of action, consistent with reports that transfection modalities (Lytle *et al*, 2007) and choice of the promoters that drive reporter gene expression (Kong *et al*, 2008) can affect the apparent mode of target repression.

Consistent with the elusive nature of miRNA mechanism(s), few molecular players have been identified. Mature miRNAs occur in a complex with Argonaute (AGO) family proteins, and it has been suggested that direct binding of AGO to the mRNA cap may be responsible for miRNA target repression (Kiriakidou *et al*, 2007), but this has been

*Corresponding author. Friedrich Miescher Institute for Biomedical Research (FMI), Maulbeerstrasse 66, WRO-1066.1.38, 4002 Basel, Switzerland. Tel.: +41 61 6976675; Fax: +41 61 6973976; E-mail: helge.grosshans@fmi.ch

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controversial (Eulalio *et al*, 2008b). The translation initiation factor eIF6 has been identified as a component of a large AGO2-containing complex in human cells and eIF6 depletion was shown to impair miRNA target gene silencing in human cells and *C. elegans* (Chendrimada *et al*, 2007). However, it has been suggested that the involvement of eIF6 may be indirect (Filipowicz *et al*, 2008), and studies of *Drosophila* cells have indicated that eIF6 may not be generally required for miRNA function (Eulalio *et al*, 2007, 2008b). Consistent with the latter notion, depletion of *C. elegans* eIF6 appears to enhance rather than diminish *let-7* miRNA activity by genetic criteria (Ding *et al*, 2008).

AGO proteins also bind to members of the GW182 protein family in various organisms and this interaction contributes to miRNA function (reviewed in Ding and Han, 2007). Tethering of GW182 to an mRNA leads to degradation of this mRNA, and, conversely, GW182 depletion impairs miRNA activity (Liu *et al*, 2005; Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2008b). In *C. elegans*, combined loss of the two GW182-like proteins AIN-1/-2 partially phenocopies loss of the AGO proteins ALG-1/-2 and causes upregulation of reporter genes under miRNA control (Ding *et al*, 2005; Zhang *et al*, 2007). The level and extent to which AIN-1/-2 contribute to miRNA function have remained unknown, although it has been suggested that they might localize repressed miRNA targets to P-bodies to enable their degradation (Ding *et al*, 2005).

We have focused here on the *C. elegans let-7* miRNA to examine the mechanism of action of miRNAs *in vivo*. *let-7* was originally identified as a component of the *C. elegans* heterochronic pathway (Reinhart *et al*, 2000), which controls the temporal fate of cells during postembryonic development. Several *let-7* target genes have been identified (Slack *et al*, 2000; Abrahante *et al*, 2003; Lin *et al*, 2003; Grosshans *et al*, 2005; Lall *et al*, 2006) and among these, *lin-41* and *daf-12* have been characterized most extensively and their *let-7*-binding sites partially mapped (Reinhart *et al*, 2000; Slack *et al*, 2000; Vella *et al*, 2004; Grosshans *et al*, 2005). This availability of *in vivo* validated targets combined with the fact that the sequence of *let-7* is perfectly conserved in animals (Pasquinelli *et al*, 2000; Lagos-Quintana *et al*, 2002), and that it has been used to examine miRNA mechanisms of action in diverse experimental systems (Bagga *et al*, 2005; Pillai *et al*, 2005; Nottrott *et al*, 2006; Mathonnet *et al*, 2007; Wakiyama *et al*, 2007), makes *let-7* particularly suitable for our analysis. In addition, understanding the mode of action of this specific miRNA is of particular interest because of its important developmental and pathological functions as a potent regulator of stem cell fates and a tumour suppressor (reviewed in Büssing *et al*, 2008).

We report that *let-7* causes repression of translation initiation as well as degradation of its endogenous *lin-41* and *daf-12* target mRNAs. Other miRNAs silence their targets by the same mechanisms, and this includes *lin-4* miRNA, previously reported to repress translation at a level after initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002). Translational repression requires the GW182 proteins AIN-1/-2, as does mRNA degradation. Our findings indicate that downregulation of translation initiation is widely used under physiological conditions in *C. elegans* and establish the nematode as a system for genetic dissection of this process.

Results

Translational blockade of endogenous let-7 target genes

We recently observed widespread genetic interaction between *let-7* and the translational machinery in *C. elegans* (Ding *et al*, 2008). These findings prompted us to examine whether *let-7* regulates its targets translationally *in vivo*. To this end, we fractionated whole animal lysates by sucrose density gradient ultracentrifugation to analyse the polyribosome association of endogenous *let-7* targets in wild-type and *let-7(n2853)* mutant *C. elegans* at the L3 developmental stage, when *let-7* activity is low, and at the late L4 stage, when *let-7* activity is high (Reinhart *et al*, 2000) (Figure 1A; Supplementary Figure S1). As the two *let-7* targets *daf-12* and *lin-41* (Slack *et al*, 2000; Grosshans *et al*, 2005) are expressed at very low levels in L4 stage larvae (Snow and Larsen, 2000; Bagga *et al*, 2005 and this study, below), we used reverse transcription–quantitative PCR (RT–qPCR) to quantify them. It is to be noted that all experiments were performed using random hexamer oligonucleotides to prime RT, to include even mRNA, the poly(A) tail of which might be short due to the action of the miRNA (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008). Additional control experiments, described below, further confirmed that we are detecting full-length mRNAs rather than partially stable degradation fragments.

We found that both *lin-41* and *daf-12* mRNAs were moderately, but consistently depleted from the highly translated polysomal fractions in wild-type relative to *let-7* mutant animals at the late L4 stage (Figure 1B and C; Supplementary Figure S1), in agreement with decreased translation initiation (Eulalio *et al*, 2008a). By contrast, *ama-1* and *act-1* mRNAs, which are not targeted by *let-7*, displayed similar translational profiles in both strains (Figure 1B and C; Supplementary Figure S1).

L3 stage animals express little or no *let-7* (Reinhart *et al*, 2000); accordingly, we see no difference when comparing polysomal association of *daf-12* and *lin-41* mRNAs between *let-7* mutant and wild-type animals at this stage (Figure 1B and C; Supplementary Figure S1). Moreover, in wild-type animals, polysome association of *daf-12* and *lin-41* is decreased at L4 compared with L3 stage, consistent with the establishment of an inhibitory mechanism affecting translation initiation as *let-7* expression starts. A more moderate decrease of polysome association is also seen when performing this comparison for *let-7* mutant animals, suggesting that the *n2853* allele may provide residual *let-7* activity or that alternative mechanisms, possibly the *let-7* sister miRNAs *mir-48*, *mir-84* and *mir-241* (Abbott *et al*, 2005; Li *et al*, 2005), may contribute.

To exclude the possibility that the RNA that we analysed in our sucrose gradients was not representative for the total pool of cellular RNA, we performed the following control experiments. We used TRIzol to extract total RNA directly from ground worms, from the cleared lysate used for sucrose gradient centrifugation and from the pellet left behind upon lysate clearing. We found, first, that ~90% of the RNA is in the lysate supernatant and will thus be loaded on the sucrose gradient. Second, composition of RNA in the supernatant and pellet is comparable, neither *let-7* target nor control mRNAs are preferentially enriched in, or depleted from, the supernatant relative to RNA retained in the pellet (Supplementary Figure S2). Finally, although increasing sucrose concentration

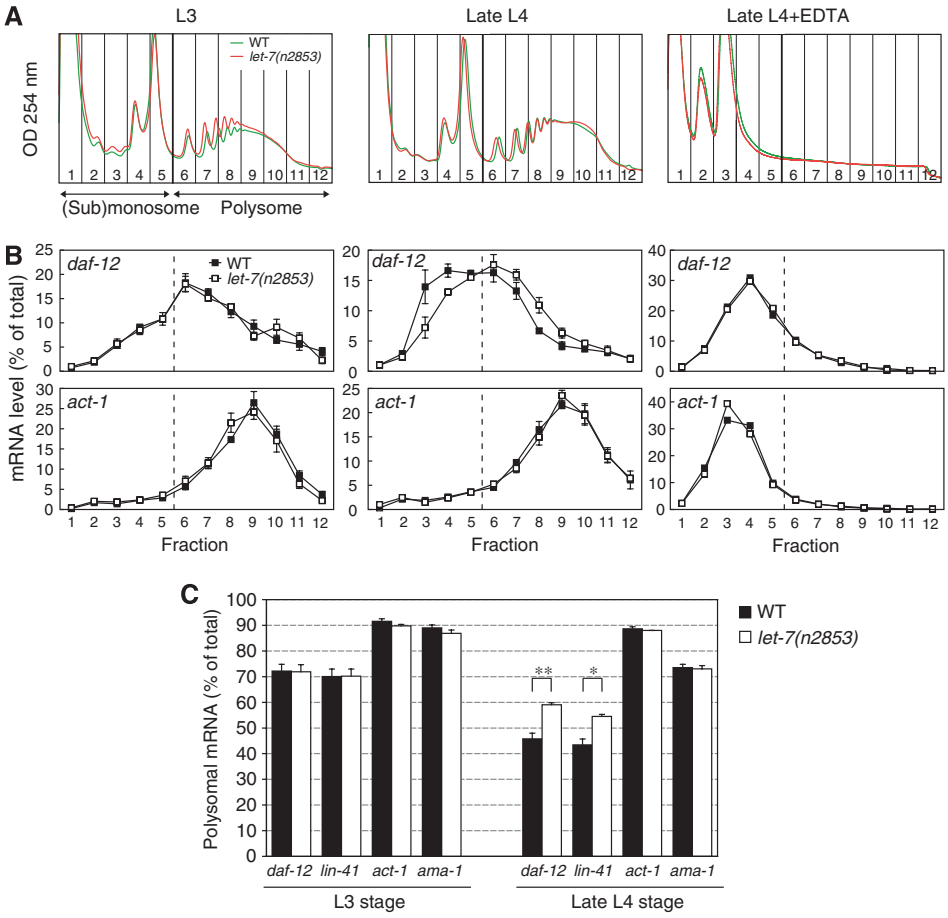

Figure 1 *let-7* decreases translation initiation on *daf-12* and *lin-41* mRNAs. (**A**) Typical polysome profile of wild-type and *let-7(n2853)* animals with or without EDTA treatment. (**B**) Distribution of *daf-12* and *act-1* mRNAs across polysome profiles from synchronized wild-type and *let-7(n2853)* animals at the L3 and late L4 stage, with or without EDTA treatment. (**C**) Polysomal fraction of *daf-12*, *lin-41*, *ama-1* and *act-1* mRNAs in L3 and late L4 wild-type and *let-7(n2853)* animals as a percentage of the total (*$P<0.05$, **$P<0.01$). mRNA levels were analysed by RT–qPCR. EDTA treatment was performed in duplicate, one representative experiment is shown. All other panels in this and subsequent figures show the averages of $n\geqslant3$ independent experiments. For this and all subsequent figures, 'WT' denotes the wild-type *N2* strain, and error bars are s.e.m.

in total lysates decreased the yield of extracted RNA, RNA composition was largely unaffected (Supplementary Figure S2). To ensure comparable recovery from each fraction and greatest possible reproducibility, we equalized sucrose concentration in all fractions to 30% (w/v) prior to RNA extractions in all our experiments. This set of control experiments confirms that any results that we obtained in our analysis can be considered representative of the total pool of cellular RNA.

To determine further whether the fast-sedimenting mRNA was indeed associated with polyribosomes, we treated lysates with EDTA and observed that all four mRNAs were shifted to the top of gradients. Distributions became indistinguishable for late L4 wild-type and *let-7(n2853)* animals (Figure 1; Supplementary Figure S1). As EDTA also disrupts non-ribosomal ribonucleoprotein complexes, we further used puromycin to disassemble specifically polysomes by inducing premature termination of the elongating peptide chains. Puromycin treatment of extracts collapsed polysomes and shifted the mRNAs deeper into the gradient (Supplementary Figure S3), possibly reflecting aggregation of the mRNA and not further pursued by us. The resulting sedimentation patterns were indistinguishable for wild-type and *let-7* animals and occurred for *let-7* target as well as control mRNAs. The coincident loss of polysomes and shift of mRNAs demonstrates that our assay examines mRNAs associated with translation-competent ribosomes.

We conclude from these data that *let-7* depletes its targets *lin-41* and *daf-12* from *bona fide* polysomes, consistent with blocking translation initiation on these mRNAs.

Translational repression requires let-7 complementary sites in the lin-41 3′UTR

The *lin-41* 3′ untranslated region (3′UTR) is necessary and sufficient to confer *let-7*-mediated regulation on an unrelated reporter gene (Slack *et al*, 2000). To verify that *let-7* impaired *lin-41* translation by binding to the *lin-41* 3′UTR, we employed transgenic animals expressing a *lacZ* reporter gene fused to the *lin-41* 3′UTR or a mutant variant thereof lacking the *let-7*-binding sites (Figure 2A). We expressed the transgene from the *col-10* promoter to accumulate it specifically in the seam cells, where *let-7* mediates *lin-41* repression (Slack *et al*, 2000; Johnson *et al*, 2003).

Consistent with inhibition of translation initiation, we observed that only 40% of the reporter mRNA was associated with polysomes in wild-type animals, whereas this level reached almost 70% in *let-7* mutant animals. Deletion of

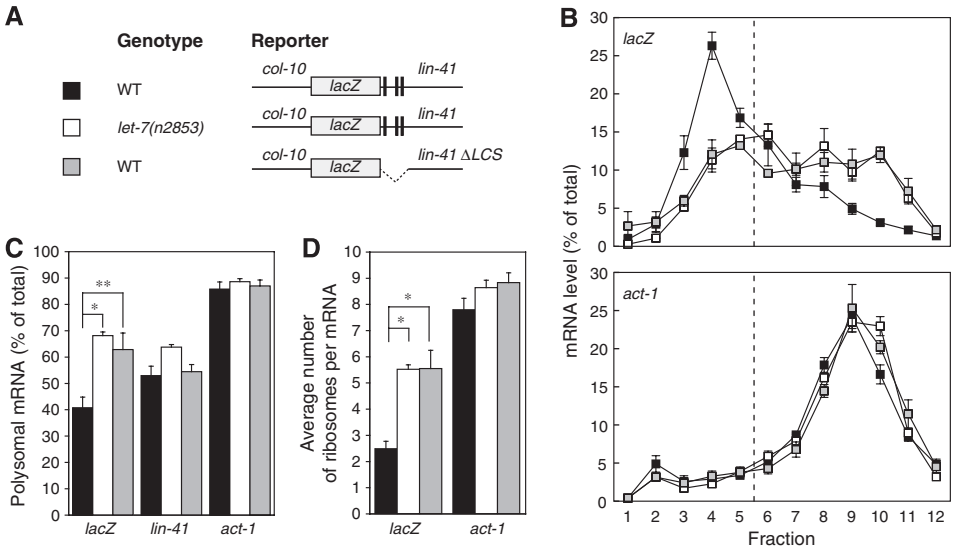

Figure 2 *lin-41* translational repression is mediated through *let-7*-binding sites. (**A**) Schematic representation of the reporter strains. Black square, WT *N2*; *Is[col-10*::*lacZ*::*lin-41]*, white square, *let-7(n2853)*; *Is[col-10*::*lacZ*::*lin-41]*, grey square, WT *N2*; *xeIs11[col-10*::*lacZ*::*lin-41-ΔLCS]*. The vertical lines in the *lin-41* 3′UTR represent *let-7* complementary sequences (LCSs). (**B**) *lacZ* and *act-1* mRNA distributions, determined by RT–qPCR, across polysome profiles. (**C**) Polysomal fraction of *lacZ*, *lin-41* and *act-1* mRNAs as a percentage of the total. (**D**) Average number of ribosomes on *lacZ* and *act-1* mRNA (*$P<0.05$, **$P<0.01$). Synchronized late L4 reporter animals were used. Note that the repression of endogenous *lin-41*, carrying the full-length 3′UTR, is maintained in the wild-type strain expressing the truncated *col-10*::*lacZ*::*lin-41-ΔLCS* transgene. The difference between endogenous *lin-41* translational repression in *let-7(n2853)* and wild-type animals is no longer statistically significant ($P=0.067$) in transgenic animals, possibly due to sequestering of endogenous *let-7* by reporter transgenes in wild-type animals.

the validated *let-7*-binding sites in the reporter 3′UTR (Vella *et al*, 2004) relieved translational repression to the same extent in wild-type animals (Figure 2B and C). Consequently, *let-7* mutation or deletion of its binding sites increased the average number of ribosomes per *lacZ* mRNA by more than two-fold relative to the wild-type situation, whereas the average number of ribosomes per *act-1* mRNA stayed constant (Figure 2D; see Materials and methods for details on the calculation). This result shows that the interaction between *let-7* and its binding sites in the *lin-41* 3′UTR mediates significant translational repression of the target mRNAs. This is confirmed by our finding that loss of *let-7* regulation causes a $\geqslant$5-fold derepression of the *lacZ* reporter (Supplementary Figure S4), although mRNA levels change less than two-fold (see below).

mRNA degradation does not correlate with translational repression

Bagga *et al* (2005) observed dramatic reduction of target mRNA levels in the presence of their cognate miRNAs in *C. elegans*. By RT–qPCR, we determined mRNA levels of the *let-7* targets in total RNA that we prepared from aliquots of the same whole animal lysates that were used for the polysome profile experiments (Figure 3A). At the L3 stage, *lin-41* and *daf-12* mRNA levels are similar in wild-type and *let-7(n2853)* animals. However, at the late L4 stage, *lin-41* mRNA is six-fold and *daf-12* two-fold more abundant in *let-7(n2853)* relative to wild-type animals. Similar ratios were obtained when summing up the amounts of these mRNAs across all fractions of the sucrose gradients, further confirming that RNA extracted from the gradients is representative of total cellular full-length mRNAs. It is to be noted that the levels of *lin-41* and *daf-12* mRNAs are reduced by two-fold even in the *let-7* mutant animals between the L3 and L4 stages.

For *lin-41*, our results are in agreement with those seen by Bagga *et al* (2005), and northern blot analysis of total RNA using a probe against *lin-41* identified a single band, the intensity of which mirrored the signal obtained by RT–qPCR in the same backgrounds (Figure 3B). Although *lin-41* mRNA levels in individual sucrose gradient fractions were below the limit of detection by northern blot analysis, these results essentially exclude the possibility that accumulation of *lin-41* mRNA degradation products could bias our RT–qPCR results and confirm that we reliably quantify full-length mRNAs.

For *daf-12* mRNA, its low abundance prevents detection by northern blotting even in unfractionated, total RNA without prior selection of polyadenylated mRNA (Snow and Larsen, 2000). Therefore, to confirm that our RT–qPCR assay similarly measures the levels of full-length *daf-12* mRNA, we tested a second set of primers, and obtained comparable results as expected (Supplementary Figure S5A). Finally, we examined the expression levels of both *daf-12* and *lin-41* using cDNA obtained through oligo-dT-primed RT. Again, we obtained comparable results (Supplementary Figure S5B and C), arguing against the detection of a stable degradation product and suggesting that any residual poly(A) tail on these mRNAs is sufficient to support priming through oligo-dT oligonucleotides. In summary, we confirm by several independent methods that our assays quantify full-length mRNAs, and we find that the *daf-12* and *lin-41* mRNAs are not only translationally repressed by *let-7* but also subject to degradation.

Translational repression of *daf-12* is at least equal to that of *lin-41* but the decrease of *daf-12* mRNA levels is more modest, suggesting that translational repression and transcript degradation may not be directly linked. Indeed, although the *lacZ*::*lin-41* reporter mRNA is very efficiently

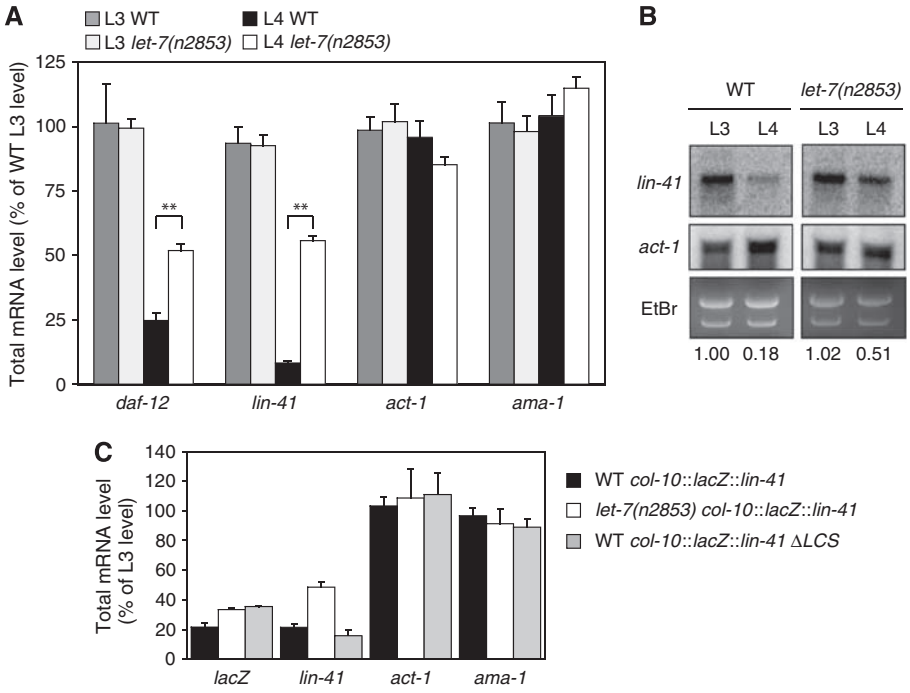

Figure 3 *let-7* mediates target mRNA degradation. (**A**) Analysis of *daf-12*, *lin-41*, *ama-1* and *act-1* total mRNA levels by RT–qPCR. Data are normalized for the average of *ama-1* and *act-1* values (**$P<0.01$). (**B**) Northern blot analysis of *lin-41* and *act-1* mRNA levels using 20 μg of total RNA. Numbers indicate *lin-41* mRNA levels normalized for *act-1*. Wild-type and *let-7(n2853)* samples are presented in separated panels for clarity; however, RNA samples were assessed on the same membrane and exposition time is identical. (**C**) Analysis of *lacZ*, *lin-41*, *act-1* and *ama-1* total mRNA levels in L4 stage reporter animals by RT–qPCR, data normalized for the average of *act-1* and *ama-1* values.

repressed translationally, mRNA levels differed by less than two-fold in wild-type relative to *let-7(n2853)* animals (Figure 3C). Although these findings strongly argue against a scenario where lower abundance of an mRNA diminishes its access to the translational machinery, we wished to exclude the possibility further that the translational effects that we observed were due to altered mRNA levels. *ugt-63* and *vit-1* are differentially expressed in synchronized late L4 wild-type and *let-7(n2853)* animals but are not direct targets of *let-7* (B Hurschler and HG, unpublished data). Although *vit-1* was four-fold less abundant in *let-7(n2853)* than in wild-type animals, and *ugt-63* was two-fold more abundant, the translational profiles of both genes were similar in wild-type and *let-7(n2853)* (Supplementary Figure S6). Thus, altered mRNA levels *per se* do not appear to influence the efficiency of translation initiation.

Multiple miRNAs function by preventing translation initiation

The finding that *let-7* mediates repression of translation initiation on its targets in *C. elegans* was unexpected, as *C. elegans lin-4* was previously reported to repress these mRNAs at a step downstream of translation initiation (Olsen and Ambros, 1999; Seggerson *et al*, 2002). To determine whether repression of translation initiation is specific for *let-7* or a more general mechanism, we tested whether *lin-4* repressed translation initiation of *lin-14* and *lin-28*, two experimentally validated targets (Wightman *et al*, 1993; Moss *et al*, 1997). *lin-4* is first expressed in the mid-L1 stage and represses *lin-14* by late L1/early L2 and *lin-28* one stage later (Olsen and Ambros, 1999; Seggerson *et al*, 2002). When we compared extracts from late L2 stage wild-type and *lin-4(e912)* mutant animals, we observed that both mRNAs were shifted into the polysomal fraction in the mutant (Figure 4A and B). This shift is particularly pronounced for *lin-28*, where the effect is highly statistically significant (Figure 4A). By contrast, polysome association of the control mRNAs *act-1* and *ama-1* and the *let-7* target *daf-12* is identical in *lin-4(e912)* and wild-type animals (Figure 4A and B). We conclude that *lin-4*, similar to *let-7*, can repress its target at the level of translation initiation.

lin-14 and *lin-28* transcript levels are increased in *lin-4* mutants compared with wild-type animals, whereas *daf-12*, *act-1* and *ama-1* mRNA levels remain unchanged (Figure 4C). The observation that *lin-4* induces a stronger translational blockade of *lin-28* than of *lin-14* and conversely a more pronounced degradation of *lin-14* than of *lin-28* further suggests that translational repression and target mRNA degradation are not directly linked mechanisms.

Translational repression and degradation of miRNA targets require the GW182 proteins AIN-1 and AIN-2

Having established that miRNAs mediate both target mRNA degradation and translational repression *in vivo*, we sought to identify the factors mediating these mechanisms. Good candidates were the GW182 homologues AIN-1 and AIN-2 (Ding *et al*, 2005; Zhang *et al*, 2007), as depletion of GW182 causes upregulation of miRNA target genes in various systems (Ding and Han, 2007). However, although mRNA degradation is readily prevented upon GW182 depletion, derepression of those miRNA targets that are not strongly regulated by degradation is typically well below that seen with AGO depletion (Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007), consistent with the proposal that GW182 proteins might

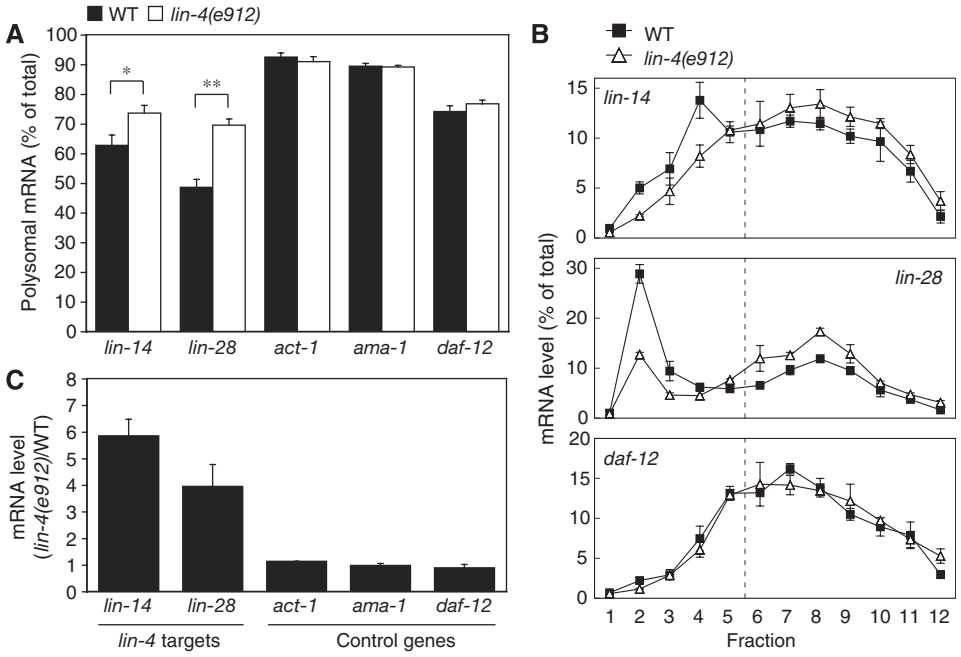

Figure 4 *lin-4* inhibits translation initiation of *lin-14* and *lin-28*. (**A**) Polysomal fraction of *lin-14*, *lin-28*, *act-1*, *ama-1* and *daf-12* in synchronized late L2 wild-type and *lin-4(e912)* animals as a percentage of the total (*$P<0.05$, **$P<0.01$). (**B**) *lin-14*, *lin-28* and *daf-12* distribution across polysome profiles from synchronized late L2 wild-type and *lin-4(e912)* animals. (**C**) Analysis of total mRNA levels in synchronized late L2 wild-type and *lin-4(e912)* animals by RT–qPCR, data are normalized for the average of the control gene values.

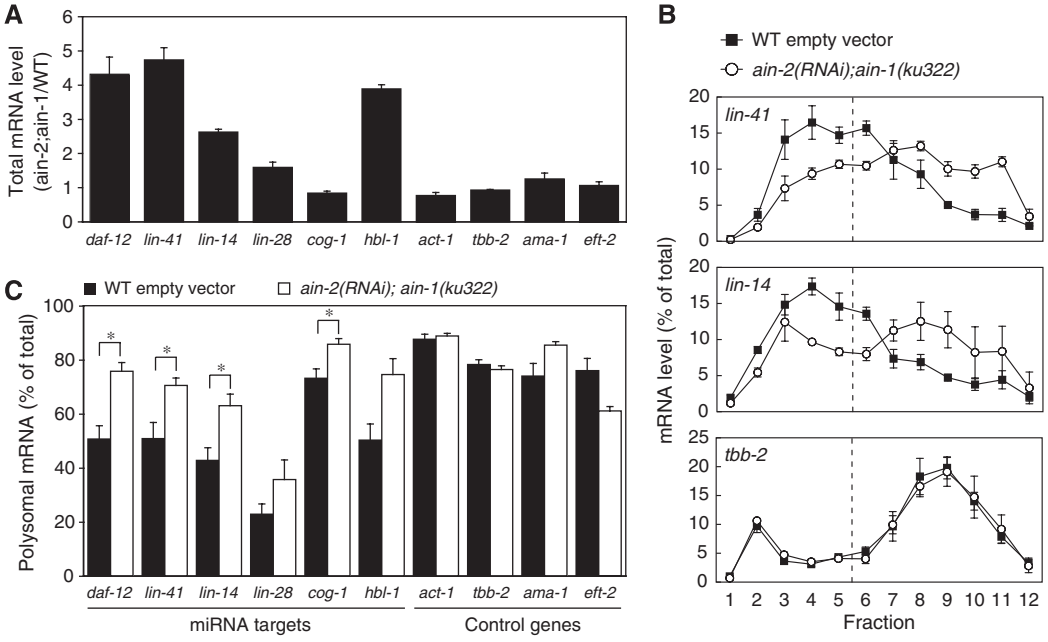

Figure 5 AIN-1 and AIN-2 mediate translational repression and degradation of miRNA target mRNAs. (**A**) Analysis of total mRNA levels in synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals by RT–qPCR, data are normalized for the average of the control gene values. (**B**) *lin-41*, *lin-14* and *tbb-2* distribution across polysome profiles from synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals. (**C**) Polysomal fraction of several miRNA targets and control genes in synchronized late L4 wild-type and *ain-2(RNAi); ain-1(ku322)* animals as a percentage of the total (*$P<0.05$).

enhance miRNA activity by targeting repressed mRNAs to P-bodies for degradation (Ding *et al*, 2005).

To determine whether depletion of the GW182 family members AIN-1 and AIN-2 permitted uncoupling of translational repression and degradation of miRNA targets, we performed polysome profile analyses on L4 stage wild-type and *ain-2(RNAi); ain-1(ku322)* animals and analysed various targets of multiple miRNAs: the *let-7* targets *daf-12* and *lin-41*, the *lin-4* targets *lin-14* and *lin-28*, the *lsy-6* targets *cog-1* (Johnston and Hobert, 2003) and *hbl-1*, which is targeted by *mir-48*, *mir-84*, *mir-241*, *let-7* and *lin-4* (Abrahante *et al*, 2003; Lin *et al*, 2003; Abbott *et al*, 2005). As predicted,

depletion of AIN-1/-2 increased *lin-41* and *daf-12* transcript levels (Figure 5A). However, to our surprise, translational repression of both *let-7* targets was also efficiently relieved (Figure 5B and C). In fact, the relief of both modes of *let-7* target repression was more extensive in *ain-2; ain-1* than in *let-7(n2853)* mutant animals, possibly suggesting that remaining *let-7* activity or distinct miRNAs, perhaps of the *let-7* family, contribute to residual repression of *lin-41* and *daf-12* in *let-7(n2853)* animals. Consistent with this idea, mRNA levels of the two *let-7* targets *daf-12* and *lin-41* are upregulated in *miR-48 miR-241; miR-84* triple mutant animals (Supplementary Figure S7).

Translational repression was also relieved for *lin-14*, *lin-28*, *cog-1* and *hbl-1*, although the results for *lin*-28 and *hbl-1* narrowly missed statistical significance (*lin-28*, $P = 0.053$; *hbl-1*, $P = 0.056$). We also analysed genes not known to be miRNA targets (*act-1*, *tbb-2*, *ama-1* and *eft-2*). We observed no effect on total mRNA levels and no consistent trend of translational upregulation in response to AIN-1/-2 depletion (Figure 5). Low abundance of the investigated miRNA target mRNAs in late L4 wild-type animals (see Figure 3B) prevented us from performing northern blot analysis on polysome profile fractions. However, consistent results were obtained by RT–qPCR with multiple *lin-14* primer pairs (Supplementary Figure S8) and by semiquantitative classical RT–PCR (Supplementary Figure S9) confirming our observation that translational repression of miRNA target is relieved in AIN-1/-2 depleted animals.

Taken together, these data reveal that translational control is a mechanism that is widely used by miRNAs *in vivo*. Equally significant, our results show that AIN-1/-2 have a general and important function in the process. Notably, although transcript levels of *lin-14*, *lin-28* and *hbl-1* increased in *ain-2; ain-1* mutant relative to wild-type animals, *cog-1* mRNA levels remained unchanged (Figure 5C), demonstrating that translational repression can occur independently of target mRNA degradation.

Discussion

We report here that endogenous *daf-12* and *lin-41* mRNAs are translationally controlled by *let-7 in vivo*. Polysomal shifts can even be seen in whole worm lysates, despite the fact that *let-7* regulates these targets only in a subset of those tissues where they are expressed (Antebi *et al*, 2000; Slack *et al*, 2000; Johnson *et al*, 2003). Nonetheless, the degree of spatial and temporal co-expression of the miRNA and its targets limits the sensitivity of our assay, as demonstrated for *pha-4*, a third experimentally validated *let-7* target (Grosshans *et al*, 2005). *let-7* regulates *pha-4* in the intestine (Grosshans *et al*, 2005), but not in the pharynx, where *pha-4* expression is particularly strong, and where *let-7* is not co-expressed (Azzaria *et al*, 1996; Johnson *et al*, 2003). Under these conditions, we can neither observe polysomal shifts (Supplementary Figure S1) nor *pha-4* mRNA accumulation (data not shown) in *let-7* mutant relative to wild-type animals. By contrast, the magnitude of repression of translation initiation can be well appreciated for the *col-10*::*lacZ*::*lin-41* reporter mRNA, which is exclusively expressed in the seam cells where *let-7* is also active.

We subsequently expanded our studies to mRNAs targeted by other miRNAs, including the *lin-4* targets *lin-14* and *lin-28* and found that these, too, were repressed at the level of translation initiation. These findings resonate well with results from cell-free and a subset of cell culture-based assays (Eulalio *et al*, 2008a; Filipowicz *et al*, 2008), and extend these studies by demonstrating such function under physiological conditions. Equally important, most published evidence for translation initiation has been obtained through the use of transfected target reporter genes, the mode of repression of which appears to be susceptible to transfection conditions (Lytle *et al*, 2007) and promoter choice (Kong *et al*, 2008). To our knowledge, only a single endogenous target gene was demonstrated to be repressed by this mechanism (Bhattacharyya *et al*, 2006), whereas this has been ruled out for others (Olsen and Ambros, 1999; Seggerson *et al*, 2002; Kong *et al*, 2008). Our study now demonstrates that repression of translation initiation is nonetheless widespread for endogenous miRNA targets, and different miRNAs.

Our finding that *lin-4* represses its targets, at least in part, at the level of translation initiation contrasts with earlier experiments that had revealed largely unchanged polysomal distributions of *C. elegans lin-14* and *lin-28* before (L1 stage) and after (L2 stage) the onset of expression of their cognate miRNA, *lin-4* (Olsen and Ambros, 1999; Seggerson *et al*, 2002). The reason for this discrepancy is currently unclear, but as *lin-4* mutant animals were not compared with wild-type animals in the earlier studies, it is possible that the resulting translational profiles might also have reflected developmental, *lin-4*-independent effects such as potential repression of *lin-28* by LIN-66 (Morita and Han, 2006). We also note that at least one of the earlier publications (Olsen and Ambros, 1999) displayed a—statistically nonsignificant—trend of *lin-14* shifting to the (sub-) monosomal fraction at the L2 stage, when *lin-4* expression is high.

Other miRNAs, in addition to *lin-4*, possibly its 'sister' *mir-237*, or even *let-7* (Reinhart *et al*, 2000; Slack *et al*, 2000; Esquela-Kerscher *et al*, 2005; Grosshans *et al*, 2005; Morita and Han, 2006), may also regulate *lin-14* or *lin-28*, and contribute to the polysomal shift observed in *ain-2; ain-1* mutant animals. However, although this remains to be tested for *lin-28*, we did not detect any change in *lin-14* mRNA levels or translation in response to the *let-7(n2853)* mutation (data not shown). Irrespective of this possibility, our experiments using *lin-4* mutant animals clearly demonstrate that this miRNA can mediate repression of translation initiation.

In most instances, we observed significant amounts of cognate mRNA degradation alongside translational repression, but the extent of degradation varied by target. Moreover, there was no clear correlation between the extent of translational repression and target mRNA degradation, for example, we observed more degradation for endogenous *lin-41* than for the reporter mRNA, although less translational silencing is apparent for the endogenous transcript. Although we cannot formally rule out the possibility that this specific case reflects differences between endogenous targets and targets expressed from transgenes or that transcriptional effects mediated by the *lin-41* promoter may contribute to these differences, we favour the idea that translational repression and target mRNA degradation may be independent mechanisms and that seam cells favour translational repression. This is consistent with an earlier study showing that different types of cultured cells evoke different responses to

identical target mRNA reporters, with degradation dominating in some cell lines and translational blockade in others (Schmitter *et al*, 2006). Indeed, the observation that *cog-1* is regulated translationally, but not at the level of mRNA degradation, further supports our conclusion that mRNA degradation and translational repression are two distinct mechanisms *in vivo* that may, however, frequently act together on the same miRNA targets.

It is to be noted that our results cannot rule out that the two mechanisms might function sequentially in that translational repression precedes mRNA degradation (e.g. Selbach *et al*, 2008). Indeed, we note that repression of translation initiation by *lin-4* appears more prominent for *lin-28* than *lin-14*. As *lin-14* is repressed at an earlier developmental stage than *lin-28* (Olsen and Ambros, 1999; Seggerson *et al*, 2002), it is tempting to speculate that increased *lin-14* mRNA degradation might deplete the monosomal pool of translationally repressed mRNA, effectively reducing the apparent polysomal shift. Analysis of polysome profiles at increased temporal resolution might help to address this possibility in the future.

Early reports on GW182 suggested a more auxiliary function in miRNA activity, with a greater importance in repressing targets susceptible to mRNA degradation (Ding *et al*, 2005; Liu *et al*, 2005; Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007). However, as AIN-1/-2 appear rather distantly related to GW182 proteins (Behm-Ansmant *et al*, 2006), it was unknown whether their functions can be inferred from GW182 activity in other organisms. Moreover, recent work on *Drosophila* GW182 has shown that degradation-independent, possibly translational, repressive mechanisms may also crucially involve GW182 (Eulalio *et al*, 2008b), possibly in an miRNA- and/or target-specific manner. We find that depletion of the GW182 proteins AIN-1/-2 severely impairs both cognate mRNA degradation and translational control *in vivo*, for a number of different miRNA targets and miRNAs, supporting the notion that these proteins are widely used, essential effectors of miRNA activities. This conclusion is also consistent with the *alg-1/2*-like phenotypes observed in *ain-2; ain-1* double mutant animals (Zhang *et al*, 2007). In view of the fact that both mRNA degradation and translational repression require AIN-1/-2, we speculate that GW182 proteins may coordinate these two activities, possibly through interaction with distinct mediators or effectors, the identities of which remain to be elucidated.

Taken together, our study provides insights into miRNA function in an animal model and establishes *C. elegans let-7* as a model for the genetic dissection of miRNA-mediated repression of translation initiation, complementing available biochemical systems. The fact that translational repression *in vivo* may be substantial at least for a subset of targets, and possibly occur even without any degradation altogether, suggests that the identification of targets of this important miRNA will benefit greatly from recently established proteomics approaches (Baek *et al*, 2008; Selbach *et al*, 2008).

Materials and methods

C. elegans strains and RNAi

Wild-type N2, MT7626: *let-7(n2853)*, MH2385: *ain-1(ku322)* and DR721: *lin-4(e912)* strains were provided by the CGC; CT5a: *N2;Is[goa-1*::*gfp; col-10*::*lacZ*::*lin-41]* (Caudy *et al*, 2003) by R Plasterk; VT1066: *mir-48 mir-241(nDf51); mir-84(n4037)* (Abbott *et al*, 2005) by V Ambros. The HW211: *let-7(n2853);Is[goa-1*::*gfp; col-10*::*lacZ*::*lin-41]* strain was obtained by crossing CT5a with MT7626. The HW390: *N2;xeIs11[rol-6(su1006); col-10*::*lacZ*::*lin-41ΔLCS 1-3]* strain was generated by genomic integration of an extrachromosomal array made of pFS1031 and *rol-6(su1006)* (Vella *et al*, 2004), followed by several rounds of backcrossing to *N2*. As *ain-2(tm1863); ain-1(ku322)* (Zhang *et al*, 2007) double mutant animals grow very poorly, we exposed *ain-1(ku322)* animals to *ain-2*(*RNAi*), starting with L1 larvae and using a published RNAi feeding construct and protocol (Kamath *et al*, 2003; Grosshans *et al*, 2005). Enhancement of the alae defect phenotype from 21% penetrant in the *ain-1(ku322)* single mutant to 100% in the double mutant confirmed efficient *ain-2* knockdown ($n \geqslant 17$ each). To circumvent reduced brood size associated with the *lin-4(e912)* allele and obtain a sufficiently large synchronized population, *lin-4(e912)* animals were expanded at 20°C on *lin-14(RNAi)*. Following synchronization by hatching in M9 buffer, animals were shifted back to standard non-RNAi food (OP50) and grown to the desired stage for extract preparation. The reappearance of the *lin-4(e912)* phenotypes (long, egg-laying defective) on these animals excluded the possibility that *lin-14(RNAi)* had prolonged effects.

Polysome profile analysis

A detailed description can be found in the Supplementary data. Briefly, lysates of synchronized worms were layered on linear sucrose gradients (15–60% w/v) and centrifuged for 3 h at 39 000 r.p.m., 4°C, using a SW-40 rotor and an Optima L-80 XP Ultracentrifuge (Beckman Coulter). The gradients were fractionated in 12 fractions of equal volume while absorbance at 254 nm was recorded. The entire gradient was fractionated so that any pelleted material would be recovered with the last fraction. However, we typically found very little RNA in this fraction, suggesting that RNAs did not substantially occur in heavy particles or compartments under our experimental conditions. After adjusting sucrose concentration in each fraction to 30% (w/v), RNA was extracted using TRIzol (Invitrogen) and RNA integrity confirmed on ethidium bromide-stained agarose gels before proceeding to RT. RNA recovery was quantitative under these conditions reaching up to 89% of input RNA. See Results section for a discussion of control experiments confirming that RNA extracted from the lysate is representative of the composition of total cellular RNA.

RT–qPCR

RNA RT was performed using the ImProm-II™ Reverse Transcription System (Promega) with random hexamer primers, according to the manufacturer's recommendations using equal amounts of RNA (400 or 800 ng) for each sample to avoid saturating the RT reactions in fractions with high concentrations of RNA. For polysome profile fractions, relative transcript levels quantified by qPCR (below) were subsequently corrected for the total amount of RNA extracted from each fraction and expressed as a percentage of the total amount recovered for the gradients. Identical results were obtained using oligo-$(dT)_{15}$ primers and equal volumes of RNA without applying any correction, validating the method and establishing that qPCR following RT by random hexamer is unlikely to detect stable degradation fragments. This was also directly examined for *lin-14* and *daf-12* using distinct qPCR primer sets, which yielded comparable results to the original primers.

qPCR reactions were performed in technical duplicate using the ABsolute™ QPCR SYBR® Green ROX Mix (Thermo Fisher Scientific), according to the manufacturer's recommendations, on an ABI Prism 7000 Sequence Detection System coupled to ABI Prism 7000 SDS 1.0 Software (Applied Biosystems). Relative transcript levels were calculated using the $2^{-\Delta C_t'}$ method (Livak and Schmittgen, 2001). For all primer pairs (Supplementary data), amplification efficiencies were determined to be equal or superior to 1.8. Control reactions lacking either the reverse transcriptase or template mRNA confirmed specificity of the amplification reaction.

Northern blot

RNA electrophoresis and transfer were performed as described earlier (Bagga *et al*, 2005). UV crosslinked membranes were hybridized using ULTRAhyb hybridization buffer (Ambion) according to the manufacturer's recommendations with randomly radiolabelled probes prepared from PCR-amplified DNA (see Supplementary data for oligonucleotide sequences). Radioactive

signals were detected and quantified using a Storage Phosphor Screen and a Typhoon 9400 with the Imagequant TL software (all GE Healthcare).

Calculation of average number of ribosomes per mRNA

To calculate the average number of ribosomes per mRNA, each gradient fraction was assigned an average number of ribosomes by counting the peaks of the polysome profile at 254 nm. This number was multiplied with relative amount of the mRNA detected in this specific fraction. The sum of this product over all the fractions yielded the average number of ribosomes per mRNA.

Statistical methods

All statistical significances were calculated using the paired one-tailed Student's *t*-test. Note that the statistical significance in this stringent test not only depends on the average and standard deviation of the data sets but also on the variation of the difference between the paired values so that error bars will not fully reflect the statistical significance obtained through this test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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