AU-Rich Element-Mediated mRNA Decay Can Occur Independently of the miRNA Machinery in Mouse Embryonic Fibroblasts and Drosophila S2-Cells

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Abstract
AU-rich elements (AREs) are regulatory sequences located in the 3′ untranslated region of many short-lived mRNAs. AREs are recognized by ARE-binding proteins and cause rapid mRNA degradation. Recent reports claimed that the function of AREs may be – at least in part – relayed through the miRNA pathway. We have revisited this hypothesis using dicer knock-out mouse embryonic fibroblasts and cultured Drosophila cells. In contrast to the published results, we find no evidence for a general requirement of the miRNA pathway in the function of AREs. Endogenous ier3 mRNA, which is known to contain a functional ARE, was degraded rapidly at indistinguishable rates in wild type and dicer knock-out mouse embryonic fibroblasts. In cultured Drosophila cells, both ARE-containing GFP reporter mRNAs and the endogenous cecA1 mRNA were resistant to depletion of the mi/siRNA factors dcr-1, dcr-2, ago1 and ago2. Furthermore, the Drosophila miRNA originally proposed to recognize AU-rich elements, mir-289, is not detectably expressed in flies or cultured S2 cells. Even our attempts to overexpress this miRNA from its genomic hairpin sequence failed. Thus, this sequence cannot serve as link between the miRNA and the AU-rich element mediated silencing pathways. Taken together, our studies in mammalian and Drosophila cells strongly argue that AREs can function independently of miRNAs.

Introduction
The output of a gene is determined by its rate of transcription, the post-transcriptional processing and stability of the mRNA, its translation rate and the post-translational control of protein activity and stability. Despite the fact that cellular mRNAs share a common set of important structural features like the 5′-cap and poly-A tail, large variations in mRNA half-life are observed, e.g. spanning from less than one hour to >24 h in mouse ES cell lines [1]. The degradation rate of mRNAs is determined by specific regulatory sequences, for which the family of AU-rich elements (AREs) is a well studied example. They were discovered in the 3′-untranslated region (3′-UTR) of unstable mRNAs coding for cytokines [2]. When transposed into an otherwise stable mRNA, AREs cause the mRNA to be deadenylated and degraded rapidly. Based on sequence differences and deadenylation kinetics, several classes of AREs have been defined [3]. Presumably, different classes of AREs recruit distinct sets of RNA-binding proteins, resulting in differential regulation. For example, Tristetraprolin (TTP) binds to class II AREs that typically occur in cytokine mRNAs and causes rapid ARE-mediated mRNA decay [4]. The destabilizing activity of TTP, however, is not constitutive: It can be temporarily masked through phosphorylation of TTP by the mitogen-activated protein kinase-activated protein kinase 2 [5]. This additional level of control helps to generate a transient peak of cytokine expression (reviewed in [6]). While in one case the recognition of an ARE by a paralog of TTP has been elucidated via crystallography [7], it is not clear how binding affinity and specificity are achieved in other cases. Because functional AREs normally contain clustered repeats of the characteristic AUUUA motif, cooperative binding of several factors in close proximity has been proposed [3].

Small non-coding RNAs such as microRNAs (miRNAs) and short interfering RNAs (siRNAs) also lead to the destabilization and translational repression of cognate mRNAs. They are bound by proteins of the Argonaute family (Ago) and confer target mRNA specificity to the RNA-induced silencing complex (RISC). Based on data obtained in Drosophila and human cell cultures, Jing et al. [8] proposed that miRNAs with appropriate sequence complementarity can recruit RISC to the ARE and thus mediate part of the repressive effect. In another report, Vasudevan and Steitz demonstrated that recognition of an ARE by miRNA-loaded RISC, in association with the Fragile-X mental retardation-related protein 1 (FXR-1), activates mRNA translation in quiescent cells [9,10]. This is surprising given that AREs and miRNAs are both generally regarded as negative regulators. Cross-talk between the miRNA and the ARE pathway has also been observed in liver cells. When grown under normal conditions,
CAT-1 mRNA is targeted by miR-122, leading to its re-localization into P-bodies and translational suppression. Upon amino acid starvation, the ARE-binding HuR protein is translocated into the cytoplasm and recruited to an ARE in the CAT-1 mRNA, downstream of the miR-122 binding site. Recruitment of HuR causes miR-122 to dissociate, whereby CAT-1 mRNA is released from P-bodies and resumes translation [11].

While most work on ARE-mediated regulation has been performed in mammalian systems, it has become clear that Drosophila melanogaster is not only capable of recognizing and degrading mRNAs containing mammalian AREs [8,12,13], but also uses this system to control endogenous transcripts, e.g. the mRNA coding for the antimicrobial peptide cecropin (cecf)A1 [14]. Moreover, the TTP-homolog tis-11 was found to be essential for this regulation in Drosophila [8,12] and overexpression of tis-11 results in a further increase of the mRNA degradation rate. We revisited the proposal by Jing et al. [9] that miRNAs are required for degradation of ARE-containing mRNAs. To this end, we examined AU-rich element mediated decay in mouse embryonic fibroblasts lacking the essential miRNA biogenesis factor dicer, and found that the half-life of endogenously expressed ier3 mRNA, an ARE-containing transcript, is unchanged. In cultured Drosophila S2 cells we observed that ARE-containing reporter mRNAs as well as endogenous cecfA1 mRNA are repressed in a tis-11 dependent manner. Again, this repression did not depend on components of the miRNA or siRNA pathways. Direct inhibition or overexpression of miRNAs with potential sequence-complementarity to the ARE did not change reporter gene expression. Finally, we demonstrate that the putative precursor of Drosophila mir-289 – the miRNA that was originally found responsible for ARE recognition in Drosophila - does not give rise to a mature miRNA. We therefore conclude that in mouse embryonic fibroblasts and Drosophila S2 cells, ARE-mediated post-transcriptional control is independent of the miRNA pathways.

Results and Discussion

Efficient ARE-mRNA decay in Dicer-deficient mouse embryonic fibroblasts

In mammalian cells, mRNAs containing class II-type AREs are rapidly degraded through binding to proteins of the TTP family. In a previous report, TTP function was suggested to depend on the microRNA miR-16 [8]. In a first approach to assess the contribution of miR-16, and miRNAs in general, to the decay activity of TTP, we used Dicer-deficient mouse embryonic fibroblasts (MEFs) [15]. These MEFs fail to produce mature miR-16 and show accumulation of the precursor pre-miR-16 (Fig. 1A). In contrast, wt MEFs show prominent expression of mature miR-16.

The mRNA encoding ier3 was identified as a target of TTP in TTP knock-out (ko) MEFs, where it is stabilized compared to wild type (wt) cells [16]. We measured decay of endogenous ier3 mRNA in both wt and dicer ko MEFs, and found that the mRNA degrades at indistinguishable rates in both cell lines (Fig. 1B and C). This result shows that neither Dicer nor miR-16 are required for ier3 mRNA decay, strongly suggesting that AREs do not generally depend on the miRNA pathway to trigger mRNA.

Artificial and authentic mammalian AREs repress GFP reporters in Drosophila cells

To validate and extend the above observation to a different system, we inserted the 3′-UTR sequences of mammalian TNF-α, IL-6 and IL-8 mRNAs [identical to the ones analyzed in [8]] downstream of the GFP coding sequence in a Drosophila expression vector [17]. In addition, we generated GFP reporter constructs containing one, two or three repeats of an artificial AU-rich sequence resembling the ARE of interferon-γ mRNA in their 3′UTRs [18] (Fig. 2A). This ARE belongs to a different subclass that has not previously been tested for its function in Drosophila [19]. Plasmids with a deletion of the ARE-sequences within the TNF-α 3′-UTR or with the TNF-α ARE inserted in the opposite orientation served as a control. Our reporters are analogous to GFP-reporters developed for assay of AU-rich element mediated decay in mammalian cells [20,21]. The primary read-out is GFP fluorescence, which can be measured by flow cytometry. We validated that the TNF-α 3′-UTR sequence destabilizes the reporter mRNA by measuring the decay after transcriptional inhibition through α-amanitin (Fig. 2A, bottom panel). Consistent with a decreased half-life, the steady-state levels of GFP-TNF-α 3′-UTR mRNA were about fourfold lower than the control mRNA before α-amanitin treatment (data not shown). We selected stable cells to avoid variations due to differences in transfection efficiency.

Figure 1. ARE-mediated mRNA decay is independent of Dicer in MEFs. A) Total RNA was extracted from wt and dicer ko MEFs, and the expression of miR-16 was examined by Northern blot analysis. dicer ko MEFs show an accumulation of the precursor pre-miR-16, whereas mature miR-16 is detected only in the wt MEFs. U6 snRNA serves as a loading control. B) Degradation of the ARE-containing ier3 mRNA was examined in wt and dicer ko MEFs. Cells were treated with 5 μg/ml Actinomycin D and RNA was isolated at the indicated time points. Then, ier3 mRNA levels were measured by quantitative real-time PCR and normalized to nucleolin mRNA levels. Shown are average values ± SD from three biological repeats. C) ier3 mRNA half-lives were calculated from the decay curves, and plotted as average values ± SD in the graph on the right side.

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Dicer MEFs

wt
ko

pre-miR-16

miR-16

U6 snRNA

0
80
160
240
320
400

0
10
20
30
40
50
60
Act. D (min)

ieron3 mRNA (%)

wt
ko

0
10
20
30
40
50
60

half life (min)

wt dicer wt dicer ko

A

B

C
that are frequently observed with transient transfection assays. Subsequently, the Drosophila TTP-homolog tis-11 was knocked down by exposing the cell lines to tis-11-specific dsRNA. The GFP signal was normalized to a non-specific control (dsRNA directed against DsRed). We observed that the GFP reporters containing two or three repeats of the artificial ARE sequence as well as the IL-6 and TNF-α 3’-UTRs were de-repressed upon knock-down of tis-11, indicating that the ARE pathway represses these mRNAs (Fig. 2B).

A discrepancy between our data and the publication by Jing et al. (2005) is that the IL-8 3’-UTR did not respond to tis-11 knock down in our hands. One possible explanation may be that the ARE-motif from this particular sequence is less accessible in our construct. It is also possible that the constitutive, high-level expression of this 3’-UTR from our construct may sequester important cellular binding factors and thus selection for stable expression might counter-select against the more responsive cells (see below). In summary, we have created GFP-based cell culture reporters that responded to depletion of tis-11 in four out of five cases.

Depletion of si/miRNA factors does not lead to de-repression of ARE reporters

If a microRNA participates in recognition of AREs, then our reporters should be de-repressed upon depletion of miRNA biogenesis factors. We exposed the stably transfected S2 cells to previously validated dsRNAs targeting dcr-1, dcr-2, ago1 and ago2 [17,22,23] to induce RNAi, then measured reporter expression (Fig. 2C). We found no consistent change of GFP levels upon impairment of the small RNA biogenesis system. While in some cases a slight de-repression by depletion of dcr-2 or ago2 was measured, these changes neither correlated with the extent of de-repression observed after tis-11 depletion, nor were they consistent with the reported effects of knocking down several components of the miRNA system [dcr-1, ago1 and ago2] in the experiments of Jing et al. [8]. In addition, we observed no change of steady-state mRNA levels for the GFP-3xARE reporter or the endogenous ARE containing cecA1 mRNA upon depletion of ago2 or dcr-2 (Fig. 2D), while depletion of tis-11 resulted in an increase of steady-state mRNA levels for both cecA1 and GFP-3xARE (Fig. 2D). We did not observe any indication for translational repression of our reporter mRNA since de-repression upon tis-11 knock down was 1.57-fold ± 0.25 for mRNA and 1.61-fold ± 0.07 for GFP fluorescence.

During the selection of stably transfected cells, only a small fraction of the original cell population is retained. It is thus possible that the properties of the selected cell population differ from the parental cells, for example resulting in a population of cells that bypasses the miRNA requirement in ARE-mediated decay. We therefore tested whether an influence of RNAi-factors on the ARE-reporters can be detected upon transient transfection of the constructs. In these experiments, we noted that prior depletion of certain cellular factors influenced the transfection efficiency (likely by inducing changes in proliferation rates). It was therefore necessary to normalize the results not only to the control RNAi treatment, but in addition to the value obtained for a control construct that did not contain an ARE. The additional normalization corrects for certain systematic variations, but introduces additional measurement noise. Upon depletion of tis-11, reporters carrying the 3’-UTR of TNF-α and IL-8 were de-repressed (Fig. 2E). A similar trend was observed for the reporters containing the IL-6 3’-UTR as well as one, two or three repeats of the artificial ARE. While de-repression of the IL-8 3’-UTR reporter occurred in the transient transfection assay, the same reporter did not respond to tis-11 knock down in the stable cells (Fig. 2B). Importantly, depletion of si/miRNA pathway components did not result in a consistent de-repression of the transiently transfected reporters (Fig. 2E): Ago1, Ago2, Dcr-1 and Dcr-2 repressed the TNF-α 3’-UTR reporter (Fig. 2E), but all other constructs remained unaffected by knockdown of mi/siRNA pathway components. Thus, it is possible that miRNAs function synergistically with ARE-sequences in certain circumstances but we found no indication for a general requirement of miRNAs for AU-rich element function. In particular, there is no indication that such a component has been consistently lost during the selection of stable cells.

Our results contradict an earlier report where the same mammalian 3’-UTRs had been employed for a pilot screen with stably transfected Drosophila cells, then the dependency on mi/ siRNA factors was validated in human HeLa cells [8]. There are several technical differences between the two studies: First, Jing et al. determined mRNA half-lives by qRT-PCR, whereas our analysis in Drosophila cells is based on reporter protein expression and steady-state mRNA levels. It is formally possible that mi/ siRNA factors destabilize ARE-containing mRNAs and at the same time increase their transcription or translation rate. Indeed, translational stimulation by miRNAs has been reported in quiescent mammalian cells [9,10]. However, such compensating effects are unlikely given that we observed the same extent of de-repression at the protein and mRNA level for our GFP-3xARE reporter.

Second, the sequences in the original publication were appended to a β-globin mRNA, whereas a fusion with GFP was used in our experiments. While the choice of reporter might account for subtle differences, it is unlikely to block ARE function completely. Insertion of heterologous or artificial sequences in our experiments – as well as authentic Drosophila AREs [14] – into the 3’-UTR of a GFP reporter led to reduced mRNA levels. Finally, GFP reporters have also been used successfully for the analysis of ARE function in mammalian cells [20], indicating that GFP is well suited as a reporter protein for the study of AU-rich element mediated decay. Insertion of miRNA target sites into the same position of the GFP reporter results in robust repression (see Fig. 3A and [17,22]), indicating that miRNA-loaded RISC can access this region of the reporter mRNA.

Finally, different promotors were used in the two studies to express the reporter genes, likely resulting in different steady-state reporter mRNA levels. Based on the detectable effects of tis-11 depletion in our system, it appears that the ARE recognition system has not been saturated. Furthermore, since the same reporter system has previously been used to detect mRNA-mediated repression [17,22], exhaustion of the small RNA response is not occurring either. Together with our results from dicer-deficient mouse embryonic fibroblasts (Fig. 1) this strongly argues that the small RNA silencing system is not universally required for mRNA degradation mediated by AU-rich elements.

Direct inhibition or overexpression of miR-289 and miR-277 does not influence ARE reporters

An alternative measure to impair mRNA mediated repression is to directly inhibit specific small RNAs using modified RNA oligonucleotides of complementary sequence [24]. Jing et al. reported that miR-289 recognizes AREs in Drosophila S2 cells [8]. We therefore treated our reporter-cell lines with 2’-O-methyl-modified RNA oligonucleotides directed against miR-289 as well as miR-277, a second miRNA that potentially base-pairs with ARE containing sequences. Neither treatment led to an increase in ARE reporter gene expression, while a miR-277 reporter construct [17]...
ARE-Recognition Does Not Generally Require miRNAs

A) Diagram showing GFP expression with and without AREs.

B) Bar graphs showing GFP expression levels under different conditions.

C) Bar graphs showing GFP expression levels under dcr-1 and ago1 RNAi conditions.

D) Bar graphs showing normalized mRNA levels under different conditions.

E) Bar graphs showing GFP expression levels under different conditions.
was strongly de-repressed by the miR-277, but not the miR-289 inhibitor (Fig. 3A). In addition, overexpression of miR-289 or miR-277 did not result in any change of GFP-ARE fluorescence (Fig. 3B).

Analysis of several deep sequencing libraries published by the modEncode consortium [25] from both S2 cells and flies did not yield any sequence that corresponded fully to mature miR-289 (in contrast to 1.5 \times 10^6 reads matching other Drosophila miRNAs). Furthermore, the current version of miRBase (http://www.mirbase.org/) has included the information from 50 deep sequencing experiments for Drosophila small RNAs and not a single read for miR-289 is annotated. For comparison, the miR-277 precursor is annotated with 340786 reads. To ensure that this observation is not an artifact due to strong, unfavorable cloning bias, we tested whether miR-289 is expressed in our S2 cells by

Figure 2. ARE mediated repression is independent of the mi/siRNA pathway in Drosophila. A) Schematic representation of the reporter constructs employed in our analysis; the sequence of one artificial ARE element is indicated 5’ to 3’ (modeled after the interferon-γ element). Bottom panel: Analysis of reporter-mRNA decay. Transiently transfected Drosophila S2-cells were treated with α-amanitin at 5 μg/ml for 0, 1 and 6 hours. Introduction of the TNF-α 3’ UTR sequence destabilizes the reporter mRNA. B) Response of the GFP reporters (stable, polyclonal cell lines) to depletion of the canonical AU-rich element binding factor tis-11. GFP fluorescence was measured by flow cytometry and normalized to control knock-down (dsRNA directed against DsRed); values are mean ± SD (n = 3). The changes were significant for 2xARE, 3xARE, IL-6 3’-UTR and TNF-α 3’-UTR when compared to the TNF-α ARE control (p<0.05, student’s t-test, n = 4). C) left panel: response of the reporters (same as in B) to depletion of dcr-1 or dcr-2; right panel: response of the reporters (same as in B) to depletion of ago1 or ago2. D) Analysis of mRNA steady-state level changes upon depletion of tis-11, ago2 and dcr-2. Quantitative RT-PCR analysis was performed for the indicated miRNAs (cecA1 = endogenous ARE target, GFP-3xARE = stable ARE reporter, rp49 = ribosomal protein mRNA), normalized to the transcript levels of gapdh, and changes were calculated relative to control RNAi directed against DsRed. The asterisk (*) indicates a significant change relative to control RNAi (p<0.03, Wilcoxon’s rank sum test, n = 6). E) Experiment analogous to B) and C) using transient transfection of the reporter constructs rather than stably expressing cells.

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Figure 3. ARE mediated repression is independent of miR-289 activity. A) Response of the GFP reporters (stable, polyclonal cell lines; miR-277 reporter: stable clonal cell line) to direct inhibition of miR-277 or miR-289; an antisense oligonucleotide directed against part of the firefly luciferase coding sequence served as control for normalization; values are the mean of two experiments. B) Response of the GFP reporters (same as in A) to overexpression of miR-277 or miR-289 (transient transfection); an unrelated plasmid (pUC18) served as control for normalization; values are the mean of two experiments. C) Northern Blot to test expression of miR-289; the same membrane was stripped and hybridized with the indicated probes.

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Northern blotting. We could neither detect mature miR-289 in control cells, whereas 5 fmol of an analogous DNA oligonucleotide were readily detected, nor in cells transfected with a vector driving expression of the putative miR-289 hairpin precursor under control of the abgRNAT promoter. In contrast to miR-289, miR-277 was readily detectable in control cells and transfection of an analogous vector expressing the miR-277 hairpin precursor resulted in strong overexpression of mature miR-277 (Fig. 5C). Taken together, we conclude that there is no evidence for the existence of mature miR-278 in Drosophila. It is therefore unclear how this sequence was identified as the miRNA responsible for ARE recognition in the publication by Jing et al. (2005). We note that miR-16, proposed by Jing et al. to recognize AREs in the mammalian system (8), is different from miR-369-3 identified by Vasudevan et al. to enhance translation of an ARE-containing reporter mRNA (10).

Conclusion

We could not reproduce an earlier observation that the miRNA pathway is required for AU-rich element mediated control using both mouse embryonic fibroblasts and Drosophila Schneider cells. Furthermore, there is no evidence that miR-289 is expressed in Drosophila. Taken together, our findings call into question the previously published model that AREs need to be recognized by miRNAs to exert their function. Our goal is to initiate a discussion and an exchange of observations that either support or disfavor a participation of small RNAs in AU-rich element mediated regulation. It should be noted that the experiments we present certainly do not exclude the possibility that AREs and miRNA binding sites may cooperate or interfere with each other in the context of a specific miRNA.

Methods

Actinomycin D chase and quantitative real-time PCR

Upon the addition of 5 µg/ml Actinomycin D (AppliChem), total RNA was extracted from the cells at different times with the GeneMATRIX Universal RNA Purification Kit and 1 µg RNA was transcribed into cDNA with Transcriptor reverse transcriptase (Roche) and Oligo(dT)18 primers. After 1:20 dilution of the resulting DNA fragment was then inserted into pKF63 using GCG GCC GCA CCA CTT CCA GCA CGT TTT T-3 prime and GCA GGA AGT ACC CGT TGC-5 prime. The resulting products were inserted into the GFP-3' at the BamHI and NotI restriction sites, replacing the GFP coding sequence with the miR-289 hairpin containing fragment.

Construction of Drosophila ARE reporter plasmids

Artificial ARE repeats were generated using the oligonucleotides 5'-GGA CAT TTT ATT ATT ATT ATT AA-3' and 5'-CTA GTA AAT ATA ATA AAA AA-3' for one ARE; 5'- GCC CAT TTT ATT ATT ATT ATT AA-3' and 5'-CTA GTA AAT ATA ATA AAA AA-3' for two AREs and 5' -GCC CAT TTT ATT ATT ATT ATT AA-3' and 5'-CTA GTA AAT ATA ATA AAA AA-3' for three AREs with NotI and XhoI compatible overhangs. After annealing, the fragments were inserted into NotI and XhoI restriction sites.

Vectors for miRNA overexpression were described previously for miR-277 (17); for miR-289 an analogous vector was prepared by amplifying a 300 nt fragment of genomic DNA surrounding the miR-277 sequence by PCR with the oligonucleotides 5'-CGCCAATATTACGTGCTGCTA-3' and 3'-AAT ATG ATG CAG ACA CGG TCA-5'. The resulting products were inserted into the GFP-3'UTR of pKF63 via the NotI and XhoI restriction sites.

Drosophila Cell Culture, RNAi, transfections and FACS analysis

Dicer-deficient and wt MEFs (15) were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS superior, Biochrom AG), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all PAN Biotech) at 37°C/5% CO2. Drosophila Schneider 2 (S2) cells (17) were cultured at 25°C in Schneider’s medium (Bio&Sell) supplemented with 10% fetal calf serum (Thermo Scientific) and Penicillin-Streptomycin mix (PA Laboratories). Poloysomal S2 cell lines with stable expression of the artificial- and authentic 3'UTR-reporters were generated as previously described (22). Transfection of reporter plasmids (100 ng or 500 ng) or cholesteryl-modified antisense 2'-O-methyl RNA oligonucleotides was performed as described (22). The antisense oligonucleotides were 5'-G-Chol-CAU CAC CUG GGC DAA AUA CUA CGA AUU GUC C-3' for luciferase control, 5'-G-Chol-UUC AGU CGC AGG CUC CAC UUA AUA UAC U-3' for as-miR-289 and 5'-G-Chol-UUC UGG CUG CCA GAU GAG UUA UCC ACU-3' for as-miR-277 (all bases were 2'-O-methyl modified). After transfection, cells were incubated for 4 days and then analyzed by flow cytometry in a Becton-Dickinson FACScalibur flow cytometer.

Depletion of individual factors was performed using previously described RNAi triggers (DsRed, dcr-1, dcr-2, ago1, ago2 (Shah et al. 2003)).
and Förstemann, 2008; Hartig, 2009). To obtain an analogous construct for tis11 the following oligonucleotides were used: 5′- TAA TAC GAC TCA TTA TAG GGA TGC AGG TAG TTT-3′ and 5′- TAA TAC GAC TCA TTA TAG GGA TGC AGG TAG TTT-3′. Cells were plated at \(1 \times 10^6\) cells/ml (500 \(\mu\)l/well) and 12 \(\mu\)g/ml dsRNA was added to the medium. On day 4, the cells were split 1:6 and the dsRNA treatment was repeated. The GFP fluorescence was quantified on day 8 by flow cytometry.

Reverse transcription and quantitative RT-PCR from Drosophila S2 cells

After treatment with dsRNA against DsRed, age2, daer-2 and tis11, RNA was extracted on day 8 with TRIZol (Invitrogen) and 100 ng RNA was used for reverse transcription with Superscript II (Invitrogen), primed with a \(dT\)\(_{16}\)-containing oligonucleotide (5′- ACGAATTCTTTTTTTTTTTTTTTTTT-3′).

Quantitative RT-PCR analysis was carried out with SYBR green mixes (DyNAmo-Flash SYBR Green qPCR Kit, Finzymes) on a TOPTical Thermocycler (Biometra). Primers used for amplification are:

- \(5′-\)CTTGTGCGGACGCCCATTT-3′ and \(5′-\)AGTGCACTTGTTGGTCTGAC-3′;
- \(5′-\)ATCCTGAGTGGGTAGGATC-3′ and \(5′-\)ATCCGTTACG-3′;
- \(5′-\)CTTCTTCAGCGACACCCATT-3′ and \(5′-\)CTTCTTCAGCGACACCCATT-3′;
- \(5′-\)CTTGTTAGGATTGTGTTTG-3′ and \(5′-\)CTTGTTAGGATTGTGTTTG-3′;
- \(5′-\)AGTCGCTCAGACCTCACTGC-3′ and \(5′-\)AGTCGCTCAGACCTCACTGC-3′;
- \(5′-\)AGT CGC AGG CTC CAC TTA AAT ATT TA-3′ and \(5′-\)AGT CGC AGG CTC CAC TTA AAT ATT TA-3′;
- \(5′-\)GATACTCTCCTTGCGCTTC-3′ and \(5′-\)GATACTCTCCTTGCGCTTC-3′;

RNA isolation from Drosophila S2-cells and detection by Northern blot

RNA was isolated from transfected S2 cells using Trizol (Invitrogen) according to manufacturer’s instructions and 3.5 \(\mu\)g of total RNA were used for Northern blot analysis as described previously [26]. The following probes were used for detection: 5′- TGT CGT CCA GAT AGT GGA TTT A-3′ for miR-277; 5′- AGT CGC AGG CTC CAC TCA ATT TA-3′ for miR-299; 5′- CAG CCT TCA AAA TGA TCT CAC T-3′ for bantam.

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Author Contributions

Conceived and designed the experiments: SH KS JS GS. Performed the experiments: SL SH KS JS GS. Analyzed the data: SH KS JS. Wrote the paper: SH KS JS.

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