Translational repression precedes mRNA deadenylation and decay in miRNA-mediated gene silencing

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Materials and Methods

DNA constructs

Luciferase reporters with synthetic and natural 3'UTRs responding to bantam miRNA (Supplementary Figure 1) were described previously (*1*). For the kinetic experiments, we transferred constructs from the original Promega copia pRL vectors into pMT-DEST48 (Invitrogen) vectors. The synthetic construct with the histone H3 (FBgn0001199) stem-loop structure (HSL) was generated by inserting the HSL sequence downstream from the 3'UTR sequence containing the bantam sites (using overlapping oligos) and was then cloned into the pMT-DEST48 vector (Figure 3E and Supplementary Figure 8). The SV40 polyadenylation site found within the vector was deleted in the histone H3 constructs using QuikChange II XL site-directed mutagenesis kit (Stratagene). The reporter construct containing the *Vha68-1* (FBgn0020368) 3'UTR sequence was generated by amplifying the 3′ UTR of the gene from *Drosophila* genomic DNA and cloning the PCR product downstream of the *Renilla* luciferase coding region (Suplementary Figure 1). To generate a *Vha68-1* 3′ UTR reporter construct that is not subject to miRNA-mediated gene silencing, 3 nucleotides in the seed region of the miRNA binding sites were altered using the QuikChange II XL site-directed mutagenesis kit (Stratagene) (Table 1). Luciferase reporter constructs with synthetic 3′ UTRs targeted by miR-9b and miR-279 were generated by ligating overlapping oligos downstream of the coding sequence for the firefly luciferase gene (Supplementary Figure 1, Table 2). Reporter constructs used in the translation stalling experiments (Figure 4 and Supplementary Figures 9-11) were created by PCR amplification of the original clones (of the various miRNA-responsive reporters and their controls) using a DNA oligo that inserts the sequence for 12 Gln, Lys or Arg residues after the 5th codon in the *Renilla* or firefly luciferase genes (Figure 4 and Supplementary Figures 10). Constructs for miRNAs (bantam, miR-9b and miR-279) and HA-tagged Argonaute 1 protein were

cloned into the pMT-Dest48 (Invitrogen) and pAc5.1(Invitrogen) plasmids for exogenous expression in S2 cells.

Cell culture, transfections and luciferase assays

Drosophila S2 cells were cultured in Express Five SFM Medium (Invitrogen) supplemented with 100 units per milliliter penicillin, 100 units per milliliter streptomycin (Cambrex BioScience) and 45 ml of 200 mM L-glutamine (Invitrogen) per 500 ml medium. All transfections were performed using Effectene reagent (Qiagen). For the steady state experiments (Figure 1A-E and Supplementary Figure 3), cells were seeded in 6-well plates 24 hours prior to transfection and were transfected in triplicate. Each well contained 0.1 µg of reporter plasmid and 0.1 µg of control plasmid. When indicated, 0.3 µg of miRNA and/or Argonaute expressing plasmids were added. In the case of control experiments (Supplementary Figure 3), 15 µg/ml of each antagomir for miR-279 and miR-9b were added to the cell medium prior to the induction of the reporter constructs. Expression of various reporters and other constructs from pMT-DEST48 (Invitrogen) plasmids was induced by addition of 500 µM copper(II)-sulfate. Samples were processed 24/48 hours after transfection with the Dual Luciferase Reporter Assay System (Promega). Samples were analyzed using a Fluorostar Optima (BMG Labtech) fluorescence plate reader.

The procedure for the kinetic experiments is shown in Supplementary Figure 4. Two 10 cm dishes (each with 15 ml of cell medium) were transfected with 0.75 µg of reporter (T - targeted or NT - non-targeted) and 0.75 µg of control plasmids. Where indicated, 1 µg of plasmid expressing the miRNA of interest was also transfected in the dish. All transfections were performed using Effectene reagent (Qiagen). Transfected S2 cells were washed twice 3 hours after transfection in a 50 ml conical tube with fresh SFM medium (Invitrogen). Two cell dishes transfected with the same plasmids were pooled and diluted to 50 ml. At this point we take a sample for the 0 hour time point. Next, expression of the reporters (and their controls) and the miRNA were induced by addition of 500 µM copper (II)-sulfate. 90 minutes after induction, cells were washed twice with medium containing 500 µM bathocuproine disulphonate (BCS), resuspended in fresh

SFM medium with 50 μM BCS and divided into 6 well plates. Samples were collected at the indicated time points and for luciferase luminescence processed by the protocol used in the Dual Luciferase Reporter Assay System (Promega). Samples were analyzed using a Fluorostar Optima (BMG Labtech) fluorescence plate reader.

RNA extraction, qRT-PCR and poly(A) tailing reactions

Total RNA was extracted from Drosophila S2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 750 μl of TRIzol reagent was used for extraction of RNA from cells in each well of 6 well plates. RNA was subsequently dissolved in diethylpyrocarbonate-treated deionized water and kept frozen at -20°C until further use. The concentration of RNA was verified by Nanodrop (OD260/280) measurement.

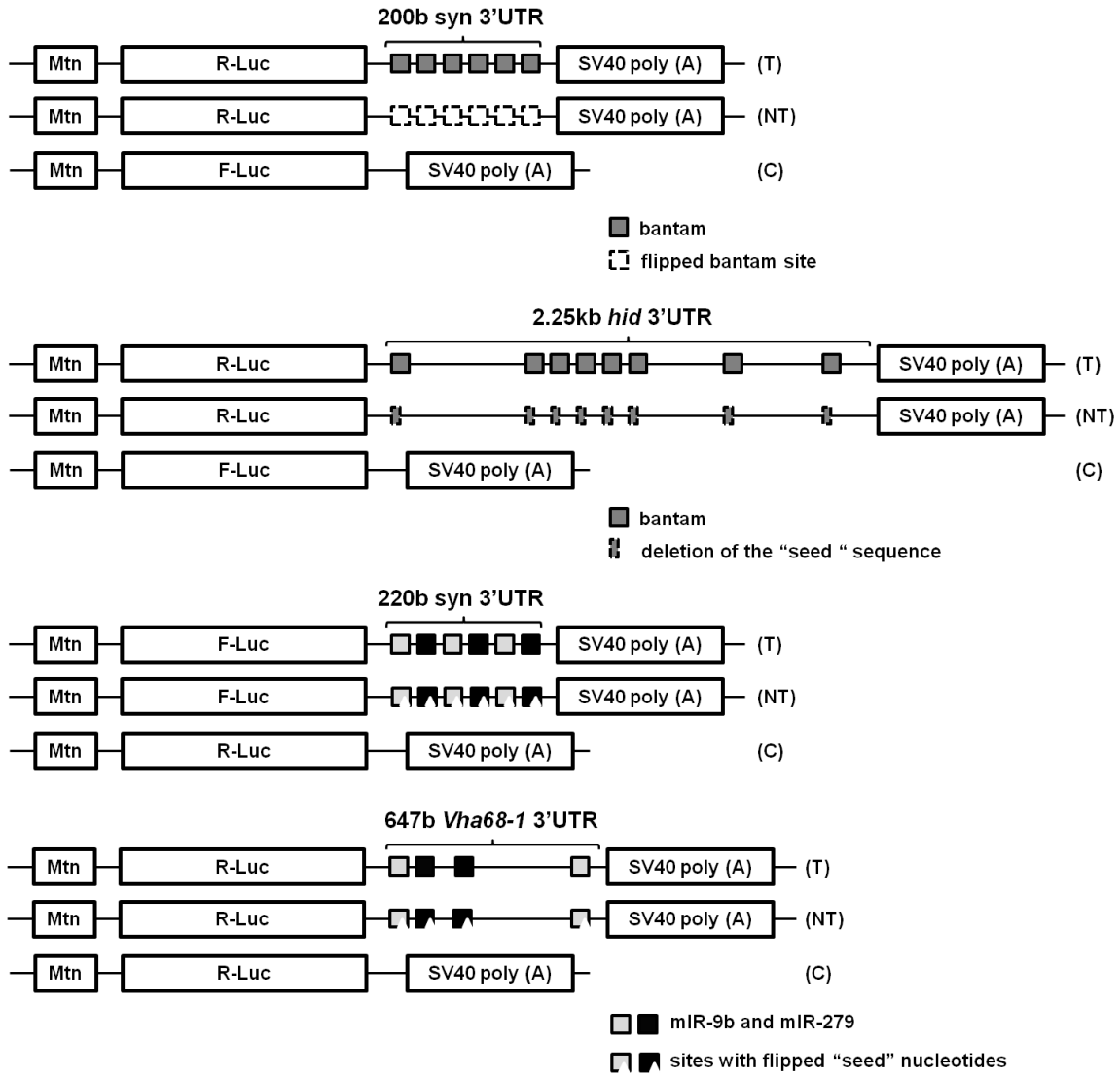
For total mRNA samples in the kinetic experiment (as well as for the samples from the translational stalling experiment), 1 μg of total RNA was used for each RT-PCR reaction following the protocol from iScript One-Step RT-PCR Kit (Biorad). qRT-PCR was done using the protocol from iQ SYBR Green Supermix (Biorad) with oligos indicated in Table 3.

Poly (A)+ samples were isolated on Dynabeads® Oligo $(dT)_{25}$ according to the manufacturer's protocol. RT-PCR and qRT-PCR for poly(A)+ samples was performed as described above using the same kits and oligos for qRT-PCR.

Length of the poly(A) tail for reporter and control RNAs was assayed using USB Poly(A) Tail-Length Assay Kit from Affymetrix according to the manufacturer's protocol. Oligos used for the PCR reaction are indicated in Table 4. PCR samples were loaded on either 2.5% or 3% agarose gels and images were recorded after standard ethidium bromide (EtBr) staining using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

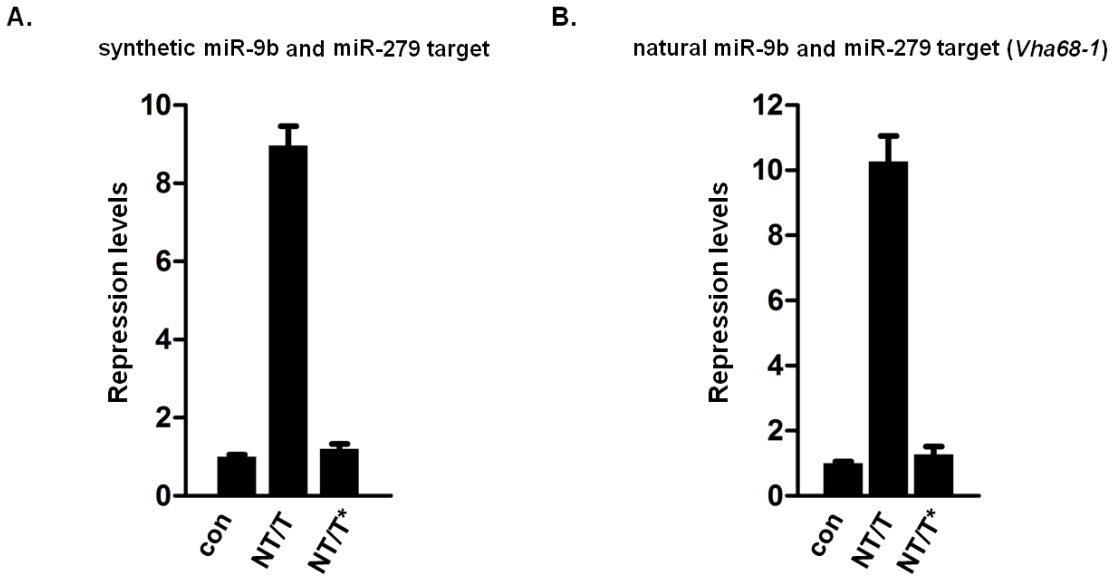
1. A. Nahvi, C. J. Shoemaker, R. Green, An expanded seed sequence definition accounts for full regulation of the hid 3' UTR by bantam miRNA. *RNA* **15**, 814 (May, 2009).

Supplementary Figure 1



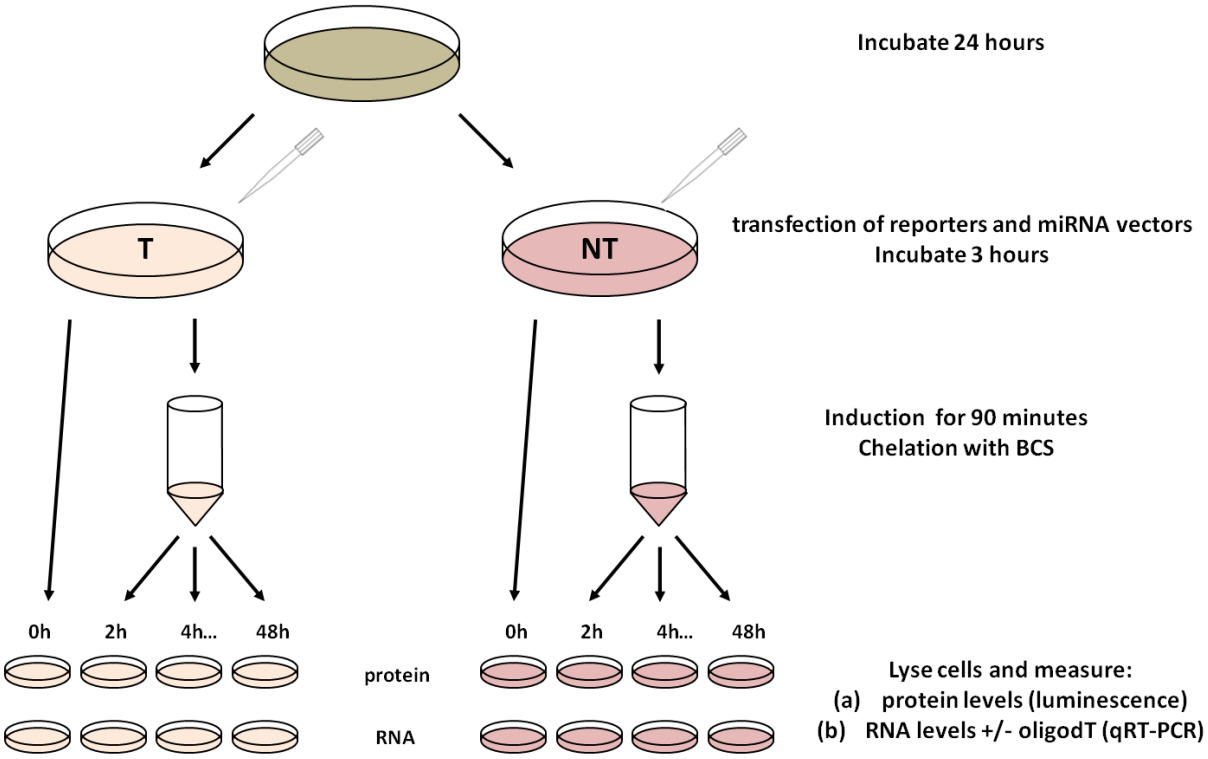
Supplementary Figure 1. Diagram of constructs used in the steady state and kinetic experiments. Synthetic 3' UTR targets were constructed from sequences that contain naturally occurring sites for bantam, miR-9b and miR-279 miRNAs. In this latter case, the miRNA binding sites were separated by a random sequence of 30 nucleotides. Natural 3'UTR targets, *hid* (FlyBAse ID: FBgn0003997) and *Vha68-1* (FBgn0020368), were amplified from a Drosophila cDNA library. Each pair of targeted (T) and non-targeted (NT) constructs is accompanied by a control (C) construct. Alterations in the miRNA binding sites are described in figure and are more explicitly detailed in XXX.

Supplementary Figure 2



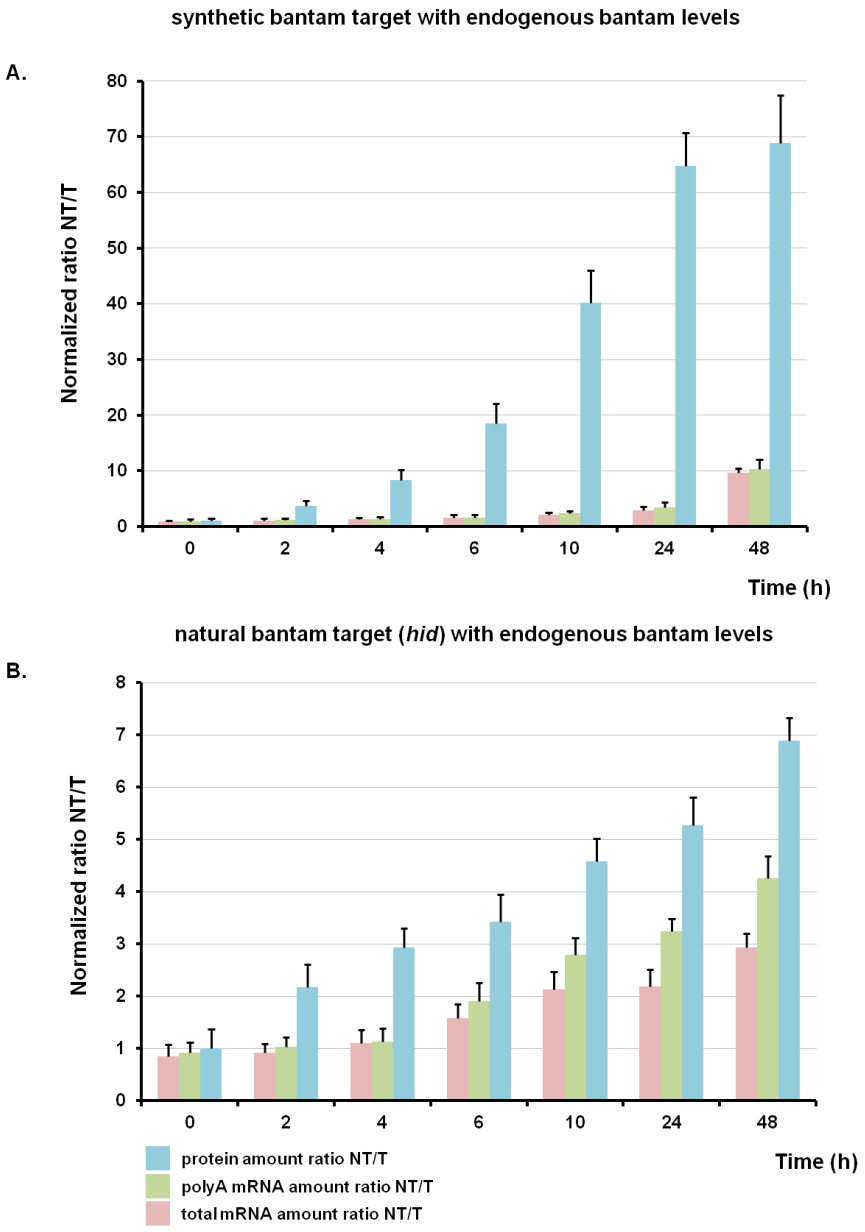
Supplementary Figure 2. Ratios of steady state protein amounts for synthetic and natural miR-9B and miR-279 targeted constructs 48 hours after reporter induction in absence (NT/T) and presence (NT/T*) of antagomirs. In each case, mean values ± standard deviation from three independent experiments are shown as a normalized ratio of protein amounts (NT/T).

Supplementary Figure 3



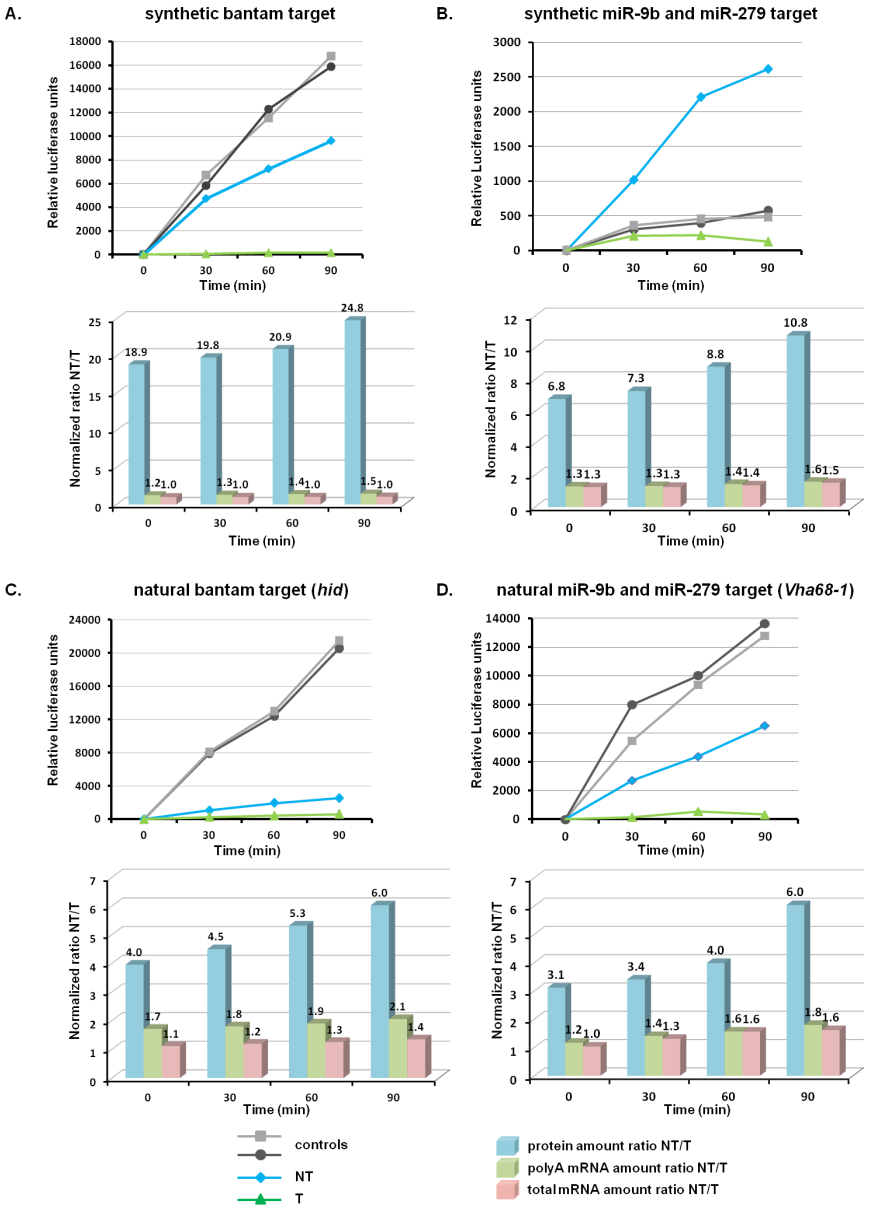
Supplementary Figure 3. Scheme of the pulse induction experiment for the kinetic analysis of miRNA-mediated gene silencing. *Drosophila* S2 cells were seeded 24 hours prior to transfection. Cells were transfected with targeted (T-orange) and non-targeted (NT-pink) reporter, control and miRNA vectors.

Supplementary Figure 5



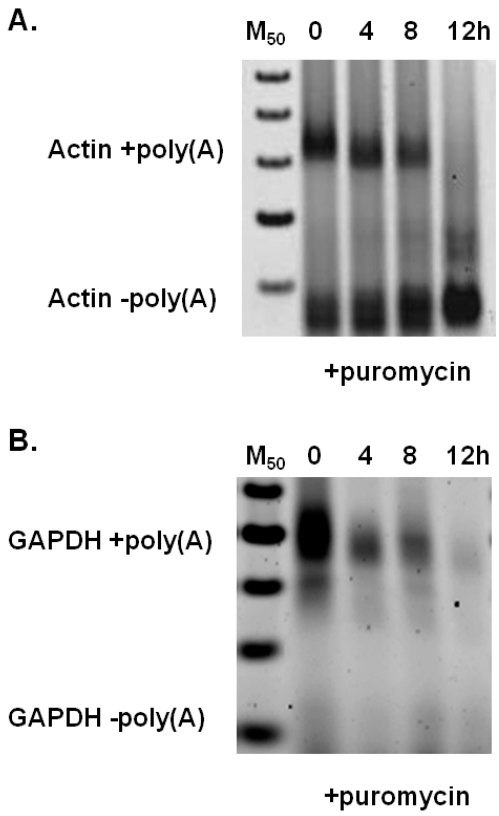
Supplementary Figure 5. (A and B) Time resolved progression of miRNA-mediated gene silencing for synthetic and natural bantam targets in presence of endogenous bantam levels. Normalized levels of protein amounts for both miRNA-targeted (T) and non-targeted (NT) constructs were used to assess the ratio of protein repression for indicated constructs. Normalized mRNA levels for reporter genes from oligo(dT)$_{25}$ resin pull down or from total RNA are presented as ratios of the amounts of poly(A) and total mRNA, respectively. Each data point represents an average value from three independent experiments. Error bars represent the mean standard deviation.

Supplementary Figure 6



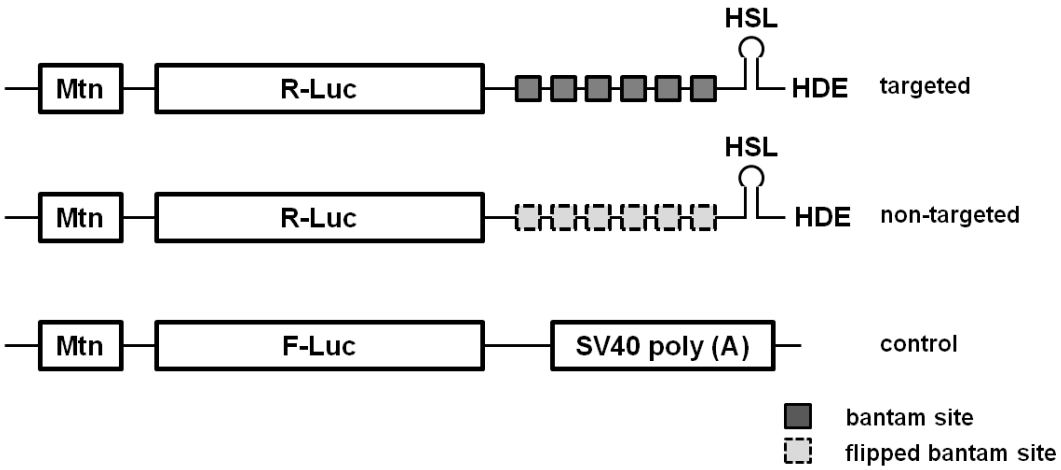
Supplementary Figure 6. (A-D) Synthesis of luciferase reporters following actinomycin D transcriptional shut-off. S2 cells were transfected with reporter, control and miRNA vectors as described for kinetic experiments. Expression of constructs was induced with addition of copper(II)-sulfate to medium for 90 minutes. Actinomycin D was added to cell media 4 hours after induction and samples were taken every 30 minutes. Diagrams above represent protein synthesis of the luciferase genes after actinomycin D treatment. Normalized levels of protein amounts for both miRNA-targeted (T) and non-targeted (NT) constructs were used to assess the ratio of protein repression for indicated constructs. Normalized mRNA levels for reporter genes from oligo(dT)$_{25}$ resin pull down or from total RNA are presented as ratios of the amounts of poly(A) and total mRNA, respectively.

Supplementary Figure 7



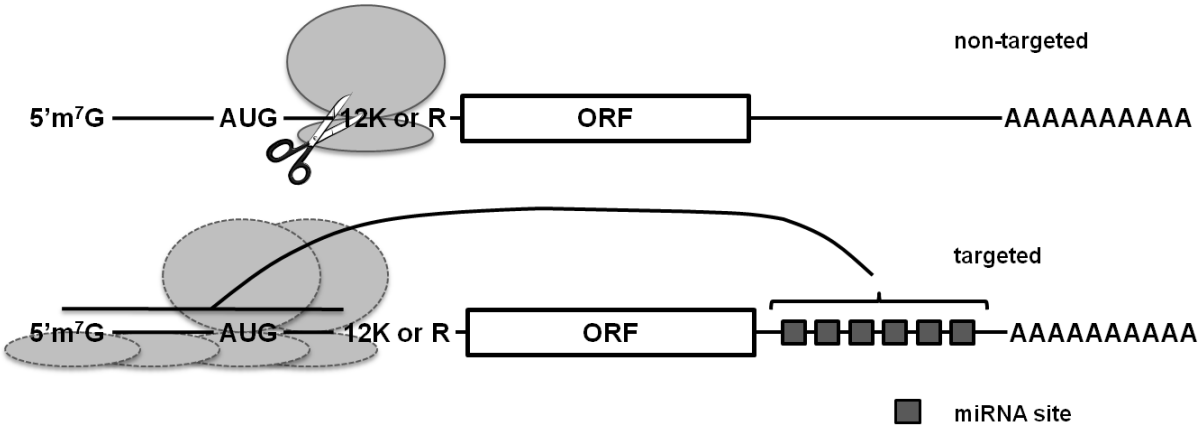
Supplementary Figure 7. Shortening of poly(A) tail in Actin 5c (FlyBase ID: FBgn0000042, A.) and GAPDH (FlyBase ID: FBgn0001091, B.) mRNAs during treatment of S2 cells with specific translational inhibitor (puromycin XXX mM). Note subtle deadenylation in 4 and 8 hour samples and more pronounced deadenylation in the 12 hour sample (Actin 5C).

Supplementary Figure 8



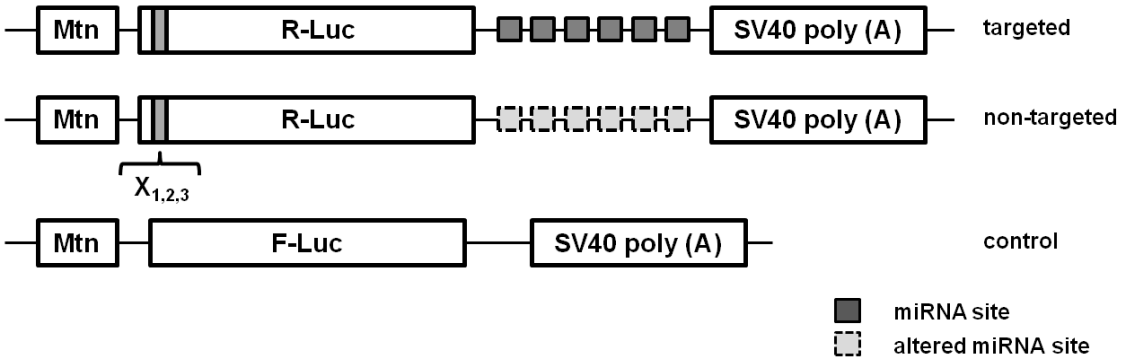
Supplementary Figure 8. Diagram of inducible reporter constructs with histone H3 terminal stem-loop (HSL) and histone downstream element (HDE) substituted for the SV40 cleavage and polyadenylation signals. Targeted, non-targeted and control constructs, as well as miRNA and altered miRNA sites are labeled respectively.

Supplementary Figure 9



Supplementary Figure 9. Schematic of translational stalling outcome for miRNA-targeted and non-targeted constructs. Scissors indicate the mRNA cleavage events induced by the translational stall. Potential translational block in miRNA-targeted mRNAs at initiation or early elongation phase is shown by flat ending arrow.

Supplementary Figure 10

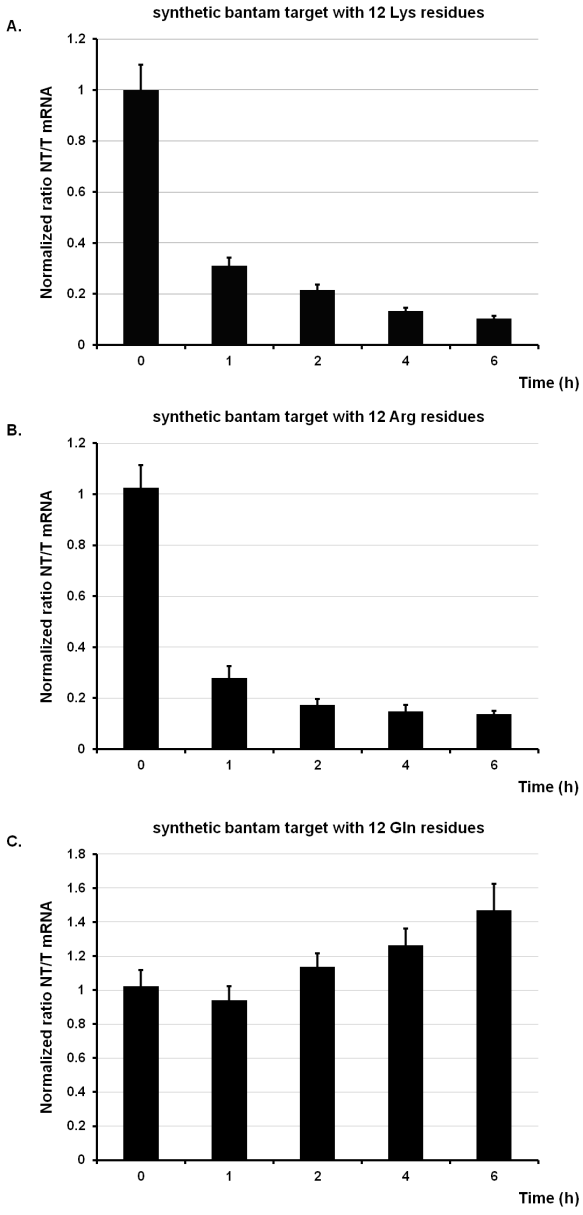


X_1	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
X_2	CGA CGA CGA CGA CGA CGA CGA CGA CGA CGA CGA CGA Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg Arg
X_3	CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln

Supplementary Figure 10. Diagram of constructs for experiments with peptide induced translational stall. X_1, X_2 and X_3 represent sequences that are introduced 5 codons after the initiating AUG start codon in

Renilla luciferase ORF. Targeted, non-targeted and control constructs are labeled according to presence

Supplementary Figure 11



Supplementary Figure 11. (A-C)Time resolved progression of miRNA-mediated effects on the stability of the mRNA of synthetic bantam target constructs containing 12 consecutive lysine- (A), arginine- (B) or glutamine- (C) residues. Both the lysine- and arginine-containing constructs lead to translational stalling and rapid mRNA decay while the glutamine-containing construct did not. Normalized mRNA amounts for the miRNA-targeted (T) and non-targeted (NT) constructs are shown as the ratio of NT/T; note that the values are now less than one and decreasing over time. Each data point represents an average of XXX measurements with standard deviation.

Table 1. Mutations (blue box) in the miRNA "seed" sequences (red box) of Vha68-1 constructs. Numbers represent position of site from initial nucleotide of Vha68-1 3'UTR sequence

miRNA site	Targeted	Non - targeted
miR-9b'	74…AGAAACCAAAGCT…	74…AGAAACCTCTGCT…
miR-279'	110…TCAATCTAGTCATA…	110…TCAATCTACAAATA…
miR-279"	197…GGTCTAGTCAGG…	197…GGTCATCTCAGG…
miR9b"	565…CTACCAAAGGA…	565…CTACCTCTGGA…

Table 2. Sequences of the synthetic miR-9B and miR279 constructs. T - targeted; NT - non targeted. Mutations in miR-9B (yellow) and miR-279 (green) are labeled by blue boxes.

construct	DNA sequence
T	CGCAAACACACACATACGCGGTCACTCAAAGCATACAATAATCCTAGTCGAGTGTGA GCTCTAGTCATATAGCCCATACAATTACAAGTCAACTGTGACCAAAGTATGCTACTCG TACAGACGAGTGTGAAATCTAGTCAAGCAGAACATAATTTCAACTTCAAAGACTCAAA GCACAAGTTCATGTACAAATGAAAATGTGTCTAGTCATAAATTGCA
NT	CGCAAACACACACATACGCGGTCACTCCCTCCATACAATAATCCTAGTCGAGTGTGA GCTGGCATCATATAGCCCATACAATTACAAGTCAACTGTGACCCGTCTATGCTACTCG TACAGACGAGTGTGAAATCTTCAGAAGCAGAACATAATTTCAACTTCAAAGACTCCCT ACACAAGTTCATGTACAAATGAAAATGTGTCTCAAGATAAATTGCA

Table 3. Oligos used for qRT-PCR experiments. Ren – Renilla luciferase, Ff- Firefly luciferase. Numbers in brackets represent position of oligo binding site from starting ATG codon of the assayed gene.

construct	Forward oligo	Reverse oligo
Ff	(473)CCAGGGATTTCAGTCGATGT	(636)AATCTCACGCAGGCAGTTCT
Ren1	(240)	

Table 4. DNA sequences of oligos used for PCR reactions in determination of poly(A) tail length

ORF	Forward oligos
Firefly luciferase	GAAAAAGAGATCGTGGATTACGTCGCC
Renilla luciferase	GGGAAAATATATCAAATCGTTCGTTG
Hid	CTGTTGTAGTTTAGTGATTGCTAGC
Vha68-1	GCTATTTCAGTAACTTGTGTGTGTTG
Actin 5C	CGAGTGGTGGAAGTTTGGAGTGCAG
GAPDH	GTTGCATAATCCGCAAGGGGCGCAAT