Supplemental Data

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Hierarchical Rules for Argonaute Loading in *Drosophila*

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Supplemental Experimental Procedures

Cell culture, transfection, and RNAi

S2-NP cells were maintained in Schneider's medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% Pen-Strep (Invitrogen, Carlsbad, CA). Cells were transfected with an expression construct for FLAG/HA-tagged AGO2 along with the selection marker plasmid pMK33-NTAP using Effectene (Qiagen, Valencia, CA). To generate a stable cell line, selection was carried out with medium containing 150 µg/mL Hygromycin B (Calbiochem, La Jolla, CA). For dsRNA treatments, ~3X10^6 cells were soaked in 1.5 mL serum-free Schneider's medium containing 10 µg dsRNAs in 6-well plates, and 3 mL serum-containing medium was added 45 minutes later. After 4 days of initial dsRNA treatment, cells were treated with dsRNAs for a second round and harvested another 4 days later as described before (Czech et al., 2008; Zhou et al., 2008). For *Renilla* luciferase reporter assays, transfection was performed in 384-well format using Effectene (Qiagen, Valencia, CA) as described before (Czech et al., 2008).

β-elimination

The chemical structure of 3' termini of small RNAs was analyzed as described (Vagin et al., 2006). In brief, RNAs from immunoprecipitates or 25 µg of total RNA from S2 cells treated with indicated dsRNAs (17 µL total volume for each sample) were incubated at room temperature for 30 min with 5 µL 5x borate buffer (148 mM borax,148 mM boric acid, pH 8.6) supplemented with 3 µL freshly prepared 200 mM $NaIO_4$. 5 µL 50% glycerol was added to quench non-reacted

sodium periodate by incubating for additional 15 min at room temperature. Samples were subsequently vacuum dried and dissolved in 60 µL 1x borax buffer (30 mM borax, 30mM boric acid, 50 mM NaOH, pH 9.5). β -elimination was carried out by incubation for 2 hours at 45°C. RNAs were Ethanol-precipitated and resolved in 1x gel loading buffer.

Northern Blotting

Northern blotting was carried out as described (Czech et al., 2008; Zhou et al., 2009). In brief, total RNAs from knockdown cells were isolated using Trizol (Invitrogen, Carlsbad, CA). 30 µg total RNAs from cultured cells (with or without β-elimination) or RNAs from immunoprecipitations were separated on 15% denaturing poly acrylamide gels and transferred to Hybond-N+ membranes (Amersham Biosciences) in 1x TBE buffer. Small RNAs were UV cross-linked to the membrane and pre-hybridized in ULTRAhyb^{TM}-Oligo buffer (Ambion, Austin, TX) for one hour. DNA probes (sequences are shown in Table S1) complementary to the indicated strands were 5' radio-labeled and added to the hybridization buffer (hybridization for 6 hours at 30°C). Membranes were washed 4 times in 1x SSC with 0.1% SDS at 30°C and exposed to PhosphorImager screens for 12-48 hours. Membranes were stripped by heating in 0.2x SSC containing 0.1% SDS in a microwave twice.

Small RNA libraries

Small RNAs were cloned as described (Brennecke et al., 2007). For this study, the following small RNA libraries from total RNAs were prepared:

19- to 24-nt from wild-type S2 cells subjected to a modified cloning strategy ("oxidized") described in (Seitz et al., 2008),

19- to 24-nt from S2 cells treated with dsRNA against *ago1*,

19- to 24-nt from S2 cells treated with dsRNA against *ago2*,

AGO1 immunoprecipitates from S2 cells treated with dsRNA against *dcr-2*, and

AGO2 immunoprecipitates from S2 cells treated with dsRNA against *dcr-2*.

Libraries were sequenced in-house or at the University of Colorado, Denver (courtesy of J. Dover and R. E. Davis) using the Illumina GAII sequencing platform. Small RNA sequences were deposited in the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE17734. In addition, we used the following published small RNA libraries for our analyses:

19- to 24-nt from wild-type S2 cells ("standard") (GSE17171) (Zhou et al., 2009),

AGO1 immunoprecipitates from S2 cells (GSE11086) (Czech et al., 2008), and

AGO2 immunoprecipitates from S2 cells (GSE11086) (Czech et al., 2008).

Bioinformatic analysis of small RNA libraries

The analysis of small RNA libraries was performed similar as described (Czech et al., 2008). Illumina reads were stripped of the 3' linker, collapsed, and the resulting small RNA sequences were matched without mismatches to the *Drosophila* release 5 genome, and to the genomes of *Drosophila* C virus, Flock house virus and Cricket paralysis virus with up to 3 mismatches. Only reads that met these conditions were subjected to further analyses. For annotations we used a combination of UCSC, miRBase, and Flybase tracks for protein coding genes, repeats/transposons, non-coding RNAs and microRNAs, as well as custom tracks (for synthetic markers, endo-siRNAs from structured loci, miR and miR* strands) with different priorities (annotation priority list available upon request). For comparison of small RNA counts between libraries, reads were normalized to the same total number after bioinformatic removal of those sequences that matched to synthetic cloning markers or assumed degradation products of abundant cellular RNAs (rRNAs, snoRNAs and tRNAs). Heatmaps were created by plotting the abundance of individual sequences within each library and by plotting the calculated relative association between the two analyzed libraries.

Supplemental Figures and Tables

Table S1. Sequences of DNA/RNA oligonucleotides

siRNA/miRNA sensor oligonucleotides	*sequence (5'->3')*
miR-276a_miR_perfect_for	TCGAAGAGCACGGTATGAAGTTCCTAAATGATATGAAGAGCACGGTATGAAGTTCCTAAATGAACTGAAGAGCACGGTATGAAGTTCCTA
miR-276a_miR_perfect_rev	TCGATAGGAACTTCATACCGTGCTCTTCAGTTCATTTAGGAACTTCATACCGTGCTCTTCATATCATTTAGGAACTTCATACCGTGCTCT
miR-276a_miR_bulged_for	TCGAAGAGCACGGTGTAAGTTCCTAAATGATATGAAGAGCACGGTGTAAGTTCCTAAATGAACTGAAGAGCACGGTGTAAGTTCCTA
miR-276a_miR_bulged_rev	TCGATAGGAACTTACACCGTGCTCTTCAGTTCATTTAGGAACTTACACCGTGCTCTTCATATCATTTAGGAACTTACACCGTGCTCT
miR-276a_star_perfect_for	TCGACGTAGGAACTCTATACCTCGCTAATGATATGACGTAGGAACTCTATACCTCGCTAATGAACTGACGTAGGAACTCTATACCTCGCT
miR-276a_star_perfect_rev	TCGAAGCGAGGTATAGAGTTCCTACGTCAGTTCATTAGCGAGGTATAGAGTTCCTACGTCATATCATTAGCGAGGTATAGAGTTCCTACG
miR-276a_star_bulged_for	TCGACGTAGGAACTACTACCTCGCTAATGATATGACGTAGGAACTACTACCTCGCTAATGAACTGACGTAGGAACTACTACCTCGCT
miR-276a_star_bulged_rev	TCGAAGCGAGGTAGTAGTTCCTACGTCAGTTCATTAGCGAGGTAGTAGTTCCTACGTCATATCATTAGCGAGGTAGTAGTTCCTACG
miR-bantam_miR_perfect_for	TCGAATCAGCTTTCAAAATGATCTCAAATGATATGAATCAGCTTTCAAAATGATCTCAAATGAACTGAATCAGCTTTCAAAATGATCTCA
miR-bantam_miR_perfect_rev	TCGATGAGATCATTTTGAAAGCTGATTCAGTTCATTTGAGATCATTTTGAAAGCTGATTCATATCATTTGAGATCATTTTGAAAGCTGAT
miR-bantam_miR_bulged_for	TCGAATCAGCTTTCCTATGATCTCAAATGATATGAATCAGCTTTCCTATGATCTCAAATGAACTGAATCAGCTTTCCTATGATCTCA
miR-bantam_miR_bulged_rev	TCGATGAGATCATAGGAAAGCTGATTCAGTTCATTTGAGATCATAGGAAAGCTGATTCATATCATTTGAGATCATAGGAAAGCTGAT
miR-bantam_star_perfect_for	TCGAGTCAAACCAAATCGAAAACCGGAATGATATGAGTCAAACCAAATCGAAAACCGGAATGAACTGAGTCAAACCAAATCGAAAACCGG
miR-bantam_star_perfect_rev	TCGACCGGTTTTCGATTTGGTTTGACTCAGTTCATTCCGGTTTTCGATTTGGTTTGACTCATATCATTCCGGTTTTCGATTTGGTTTGAC
miR-bantam_star_bulged_for	TCGAGTCAAACCAAGAGAAAACCGGAATGATATGAGTCAAACCAAGAGAAAACCGGAATGAACTGAGTCAAACCAAGAGAAACCGG
miR-bantam_star_bulged_rev	TCGACCGGTTTTCTCTTGGTTTGACTCAGTTCATTCCGGTTTTCTCTTGGTTTGACTCATATCATTCCGGTTTTCTCTTGGTTTGAC
Xba-si2-S2-2P-F	CTAGACAACAGTTTATTGGAGCGAACTTGTTGGAGTCAAAATGAACTGAGGGTGGAGCGAACTTGTTGGAGTCAAC
Xho-si2-S2-2P-R	TCGAGTTGACTCCAACAAGTTCGCTCCACCCTCAGTTCATTTTGACTCCAACAAGTTCGCTCCAATAAACTGTTGT

Oligonucleotides for pri-miRNA cloning	***sequence (5'->3')***
EcoR-Bantam-F	GGAATTCTTTTGAGTCTCTCGTTC
Bam-Bamtam-R	GGGGATCCTCAAAACCTTTGAACCTTTTAAATCC
EcoR-miR-276a-F	GGAATTCTGAAGAGAATCCCATCAGTC
Bam-miR-276a-R	GGGGATCCAAATATCATCGCATTTCGCTC

Probes for Northern blotting	***sequence (5'->3')***	***Notes***
probe-miR276a-WT-F	CGTAGGAACTCTATACCTCGCT	probe *miR-276a-1* top strand
probe-miR276a-WT-R	AGAGCACGGTATGAAGTTCCTA	probe *miR-276a-1* bottom strand
probe-miR276a-guide-5A-F	CGAAGGAACTCTATACCTCGCT	probe *miR-276a-2* top strand
probe-miR276a-guide-5A-R	AGAGCACGGTATGAAGTTCCTT	probe *miR-276a-2* bottom strand
probe-miR276a-star-5U-F	CGTAGGAACTCTATACCTCGCA	probe *miR-276a-3* top strand
probe-miR276a-star-5U-R	AGTGCACGGTATGAAGTTCCTA	probe *miR-276a-3* bottom strand
probe-miR276a-guide-5A-star-5U-F	CGAAGGAACTCTATACCTCGCA	probe *miR-276a-4* top strand
probe-miR276a-guide-5A-star-5U-R	AGTGCACGGTATGAAGTTCCTT	probe *miR-276a-4* bottom strand
probe-miR276a-central-seed-guide-5A-star-5U-F	CGAAGAAACTCTATACCTCGCA	probe *miR-276a-5* top strand
probe-miR276a-central-seed-guide-5A-star-5U-R	AGTGCGAGGTTAAGAGTACCTT	probe *miR-276a-5* bottom strand
probe-miR276a-central-guide-5A-star-5U-F	CGAAGGAACTCTATACCTCGCA	probe *miR-276a-6* top strand
probe-miR276a-central-guide-5A-star-5U-R	AGTGCGAGGTTAAGAGTTCCTT	probe *miR-276a-6* bottom strand
probe-miR276a-seed fixed-guide-5A-star-5U-F	CGAAGGAACTCTATACCTCGCA	probe *miR-276a-7* top strand
probe-miR276a-seed fixed-guide-5A-star-5U-R	AGTGCGAGGTATGTAGTTCCTT	probe *miR-276a-7* bottom strand
probe-miR276a-central-F	CGTAGGAACTCTATACCTCGCT	probe *miR-276a-8* top strand
probe-miR276a-central-R	AGAGCGAGGTTAAGAGTTCCTA	probe *miR-276a-8* bottom strand
probe-miR276a-seed-F	CGTAGAAACTCTATACCTCGCT	probe *miR-276a-9* top strand
probe-miR276a-seed-R	AGAGCGAGGTATAGAGTACCTA	probe *miR-276a-9* bottom strand
probe-miR276a-central-seed-F	CGTAGAAACTCTATACCTCGCT	probe *miR-276a-10* top strand
probe-miR276a-central-seed-R	AGAGCGAGGTTAAGAGTACCTA	probe *miR-276a-10* bottom strand
probe-miR276a-seed only-F	CGTAGGAACTCTATACCTCGCT	probe *miR-276a-11* top strand
probe-miR276a-seed only-R	AGAGCACGGTATAGAGTTCCTA	probe *miR-276a-11* bottom strand
probe-mod(let7)_same ends-F	AATTTACAACCCGGCTACCAAA	probe *let-7-1* top strand
probe-mod(let7)_same ends-R	AATTTGGTAGCCGGGTTGTAAA	probe *let-7-1* bottom strand
probe-mod(let7)_same ends+guide bulge-F	AATTTACAACCCTGCTACCAAA	probe *let-7-2* top strand
probe-mod(let7)_same ends+guide bulge-R	AATTTGGTAGTCGGGTTGTAAA	probe *let-7-2* bottom strand
probe-mod(let7)_same ends+pas bulge-F	AATTTACAACTCGGCTACCAAA	probe *let-7-3* top strand
probe-mod(let7)_same ends+pas bulge-R	AATTTGGTAGCCTGGTTGTAAA	probe *let-7-3* bottom strand
probe-mod(let7)_unstable guide-F	AATTTACAACCCGGCTACCATA	probe *let-7-4* top strand
probe-mod(let7)_unstable guide-R	AAATTGGTAGCCGGGTTGTAAA	probe *let-7-4* bottom strand
probe-mod(let7)_unstable guide+guide bulge-F	AATTTACAACCCTGCTACCATA	probe *let-7-5* top strand
probe-mod(let7)_unstable guide+guide bulge-R	AAATTGGTAGTCGGGTTGTAAA	probe *let-7-5* bottom strand
probe-mod(let7)_unstable guide+pas bulge-F	AATTTACAACTCGGCTACCATA	probe *let-7-6* top strand
probe-mod(let7)_unstable guide+pas bulge-R	AAATTGGTAGCCTGGTTGTAAA	probe *let-7-6* bottom strand
probe-mod(let7)_unstable pas-F	AAATTACAACCCGGCTACCAAA	probe *let-7-7* top strand
probe-mod(let7)_unstable pas-R	AATTTGGTAGCCGGGTTGTATA	probe *let-7-7* bottom strand
probe-mod(let7)_unstable pas+guide bulge-F	AAATTACAACCCTGCTACCAAA	probe *let-7-8* top strand
probe-mod(let7)_unstable pas+guide bulge-R	AATTTGGTAGTCGGGTTGTATA	probe *let-7-8* bottom strand
probe-mod(let7)_unstable pas+pas bulge-F	AAATTACAACTCGGCTACCAAA	probe *let-7-9* top strand
probe-mod(let7)_unstable pas+pas bulge-R	AATTTGGTAGCCTGGTTGTATA	probe *let-7-9* bottom strand
miR-bantam	AATCAGCTTTCAAAATGATCTCA	
miR-bantam-star	AGTCAAACCAAATCGAAAACCGG	
miR-184	GCCCTTATCAGTTCTCCGTCCA	
miR-184-star	ACGGGGCGAGAGAATGATAAGG	
miR-276a	AGAGCACGGTATGAAGTTCCTA	
miR-276a-star	CGTAGGAACTCTATACCTCGCT	
esi-2.1-guide	GGAGCGAACTTGTTGGAGTCAA	
esi-2.3-guide	TGACTTCCGGCGGTTAAGATTT	
2S rRNA	TACAACCCTCAACCATATGTAGTCCAAGCA	

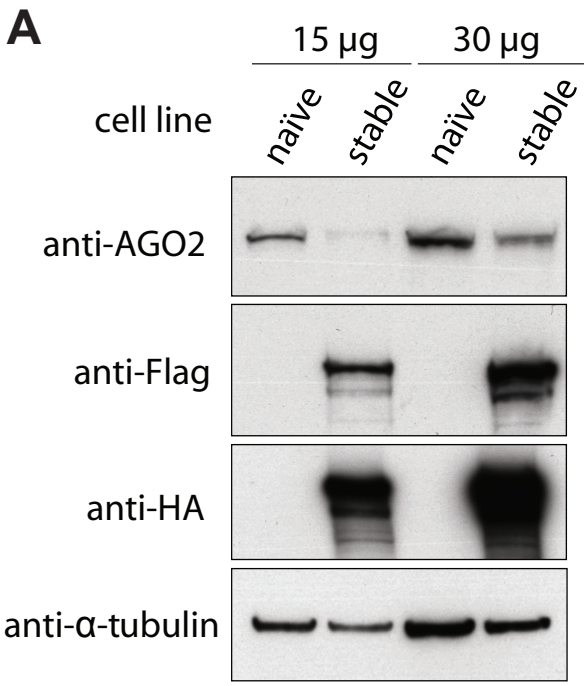
siRNAs for Argonaute loading assay	***sequence (5'->3')***	***Notes***
miR276a-WT-F	agcgagguauagaguuccuacg	*miR-276a-1* top strand
miR276a-WT-R	uaggaacuucauaccgugcucu	*miR-276a-1* bottom strand
miR276a-guide-5A-F	agcgagguauagaguuccuUcg	*miR-276a-2* top strand
miR276a-guide-5A-R	Aaggaacuucauaccgugcucu	*miR-276a-2* bottom strand
miR276a-star-5U-F	Ugcgagguauagaguuccuacg	*miR-276a-3* top strand
miR276a-star-5U-R	uaggaacuucauaccgugcAcu	*miR-276a-3* bottom strand
miR276a-guide-5A-star-5U-F	UgcgagguauagaguuccuUcg	*miR-276a-4* top strand
miR276a-guide-5A-star-5U-R	AaggaacuucauaccgugcAcu	*miR-276a-4* bottom strand
miR276a-central-seed-guide-5A-star-5U-F	UgcgagguauagaguuCuUcg	*miR-276a-5* top strand
miR276a-central-seed-guide-5A-star-5U-R	AaggUacuCUUAaccUCgcAcu	*miR-276a-5* bottom strand
miR276a-central-guide-5A-star-5U-F	UgcgagguauagaguuccuUcg	*miR-276a-6* top strand
miR276a-central-guide-5A-star-5U-R	AaggaacuCUUAaccUCgcAcu	*miR-276a-6* bottom strand
miR276a-seed fixed-guide-5A-star-5U-F	UgcgagguauagaguuccuUcg	*miR-276a-7* top strand
miR276a-seed fixed-guide-5A-star-5U-R	Aaggaacuacauaccucgcacu	*miR-276a-7* bottom strand
miR276a-central-F	agcgagguauagaguuccuacg	*miR-276a-8* top strand
miR276a-central-R	uaggaacuCUUAaccUCgcucu	*miR-276a-8* bottom strand
miR276a-seed-F	agcgagguauagaguuUcuacg	*miR-276a-9* top strand
miR276a-seed-R	uaggUacuCUauaccUCgcucu	*miR-276a-9* bottom strand
miR276a-central-seed-F	agcgagguauagaguuUcuacg	*miR-276a-10* top strand
miR276a-central-seed-R	uaggUacuCUUAaccUCgcucu	*miR-276a-10* bottom strand
miR276a-seed only-F	agcgagguauagaguuccuacg	*miR-276a-11* top strand
miR276a-seed only-R	uaggaacuCUauaccgugcucu	*miR-276a-11* bottom strand
mod(let7)_same ends-F	UUUGGUAGCCGGGUUGUAAAUU	*let-7-1* top strand
mod(let7)_same ends-R	UUUACAACCCGGCUACCAAAUU	*let-7-1* bottom strand
mod(let7)_same ends+guide bulge-F	UUUGGUAGCAGGGUUGUAAAUU	*let-7-2* top strand
mod(let7)_same ends+guide bulge-R	UUUACAACCCGACUACCAAAUU	*let-7-2* bottom strand
mod(let7)_same ends+pas bulge-F	UUUGGUAGCCGAGUUGUAAAUU	*let-7-3* top strand
mod(let7)_same ends+pas bulge-R	UUUACAACCAGGCUACCAAAUU	*let-7-3* bottom strand
mod(let7)_unstable guide-F	UAUGGUAGCCGGGUUGUAAAUU	*let-7-4* top strand
mod(let7)_unstable guide-R	UUUACAACCCGGCUACCAAUUU	*let-7-4* bottom strand
mod(let7)_unstable guide+guide bulge-F	UAUGGUAGCAGGGUUGUAAAUU	*let-7-5* top strand
mod(let7)_unstable guide+guide bulge-R	UUUACAACCCGACUACCAAUUU	*let-7-5* bottom strand
mod(let7)_unstable guide+pas bulge-F	UAUGGUAGCCGAGUUGUAAAUU	*let-7-6* top strand
mod(let7)_unstable guide+pas bulge-R	UUUACAACCAGGCUACCAAUUU	*let-7-6* bottom strand
mod(let7)_unstable pas-F	UUUGGUAGCCGGGUUGUAAUUU	*let-7-7* top strand
mod(let7)_unstable pas-R	UAUACAACCCGGCUACCAAAUU	*let-7-7* bottom strand
mod(let7)_unstable pas+guide bulge-F	UUUGGUAGCAGGGUUGUAAUUU	*let-7-8* top strand
mod(let7)_unstable pas+guide bulge-R	UAUACAACCCGACUACCAAAUU	*let-7-8* bottom strand
mod(let7)_unstable pas+pas bulge-F	UUUGGUAGCCGAGUUGUAAUUU	*let-7-9* top strand
mod(let7)_unstable pas+pas bulge-R	UAUACAACCAGGCUACCAAAUU	*let-7-9* bottom strand

Table S2. Fly strains used or produced in this study

genotype	*source*	*parental stock*
yw,hs-flp;arm-LacZ,FRT40A/CyO	This study	Bloomington
yw,hs-flp;arm-LacZ,FRT42D	This study	Bloomington
yw,hs-flp;arm-LacZ,FRT80B	Bloomington	
yw,hs-flp;arm-LacZ,FRT82B/TM6,Tb	This study	Bloomington
yw ey-flp;FRT82B dcr-1^{Q1147X}/TM3,Sb,Ser	(Lee et al., 2004)	
yw ey-flp;FRT42D dcr-2^{L811fsX}	(Lee et al., 2004)	
w;FRT80B,ago2^{414}	This study	(Okamura et al, 2004)
FRT42D,ago1^{EMS1}/CyO	This study	(Yang et al., 2007)
FRT40A,loqs^{KO}/CyO	(Park et al., 2007)	
FRT40A,r2d2^{1}/CyO	This study	(Liu et al., 2003)
tub-EGFP-bantam-guide-perfect	(Brennecke et al., 2003)	
tub-EGFP-bantam-guide-bulge	This study	
tub-EGFP-bantam-star-perfect	This study	
tub-EGFP-bantam-star-bulge	This study	
tub-EGFP-miR276a-guide-perfect	This study	
tub-EGFP-miR276a-guide-bulge	This study	
tub-EGFP-miR276a-star-perfect	This study	
tub-EGFP-miR276a-star-bulge	This study	
w;[FLAG/HA-AGO2]/CyO	(Czech et al., 2008)	

Figure S1. AGO2 expression in naïve and FLAG/HA-AGO2 cells and in the wing imaginal disc.

(A) Cell extracts from naïve S2 cells or FLAG/HA-AGO2 stable cells expressing tagged AGO2 under its endogenous regulatory elements (Czech et al., 2008) were subjected to Western blotting using antibodies against AGO2, the FLAG and HA epitope and α-tubulin as control for equal loading. Note that each panel was from identical sets of samples processed in parallel using the corresponding antibodies. Our stable cell line expresses less total AGO2 than do naïve S2 cells. **(B)** Expression pattern of FLAG/HA-AGO2 in the wing imaginal disc of transgenic flies was examined by immunofluorescence using an anti-HA antibody. FLAG/HA-AGO2 shows uniform, broad cytoplasmic expression.


A
15 µg
30 µg
cell line
naive
stable
naive
stable
anti-AGO2
anti-Flag
anti-HA
anti-α-tubulin

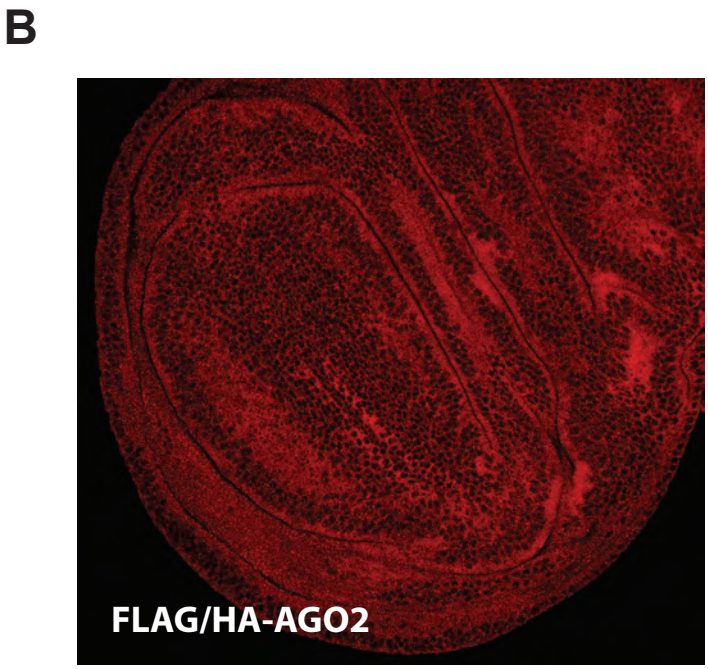

B
FLAG/HA-AGO2

Figure S2. Duplex structures for miRNAs and endo-siRNAs.

Shown are the miRNA and endo-siRNA sequences and their duplex structures that were detected by Northern Blotting in Figure 2B. The miR or guide strand (shown in red) is annealed to its corresponding miR* or passenger strand (shown in blue) with Watson-Crick base pairs indicated by lines and GU wobble base pairs as colons.

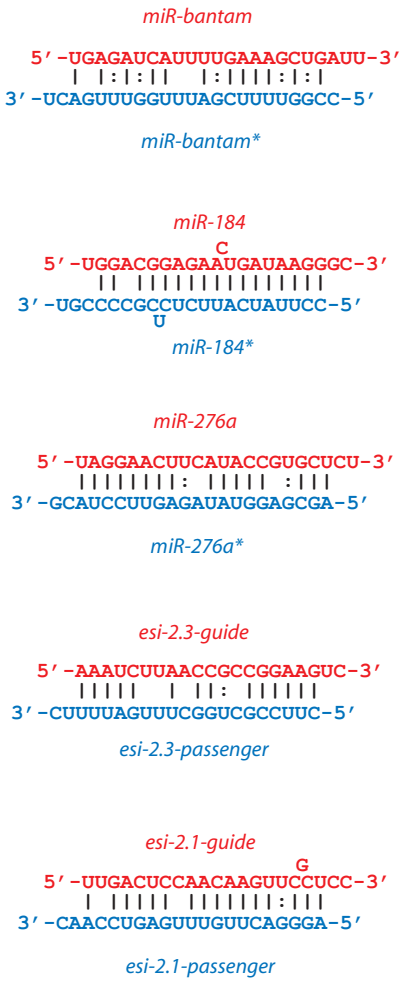

miR-bantam
5′-UGAGAUCAUUUUGAAAGCUGAUU-3′
3′-UCAGUUUGGUUUAGCUUUUGGCC-5′
miR-bantam*
miR-184
C
5′-UGGACGGAGAAUGAUAAGGGC-3′
3′-UGCCCCGCCUCUUACUAUUCC-5′
U
miR-184*
miR-276a
5′-UAGGAACUUCAUACCGUGCUCU-3′
3′-GCAUCCUUGAGAUAUGGAGCGA-5′
miR-276a*
esi-2.3-guide
5′-AAAUCUUAACCGCCGGAAGUC-3′
3′-CUUUUAGUUUCGGUCGCCUUC-5′
esi-2.3-passenger
esi-2.1-guide
G
5′-UUGACUCCAACAAGUUCCUCC-3′
3′-CAACCUGAGUUUGUUCAGGGA-5′
esi-2.1-passenger

Figure S3. AGO loading pattern of individual strands of various modified *let-7* duplexes.

Immunoprecipitation followed by Northern blotting shows the loading of both top (guide) and bottom (passenger) strands for various modified *let-7* duplexes into AGO1 or AGO2. *miR-bantam* and *esi-2.1* served as controls for AGO1 and FLAG-AGO2 immunoprecipitation, respectively.

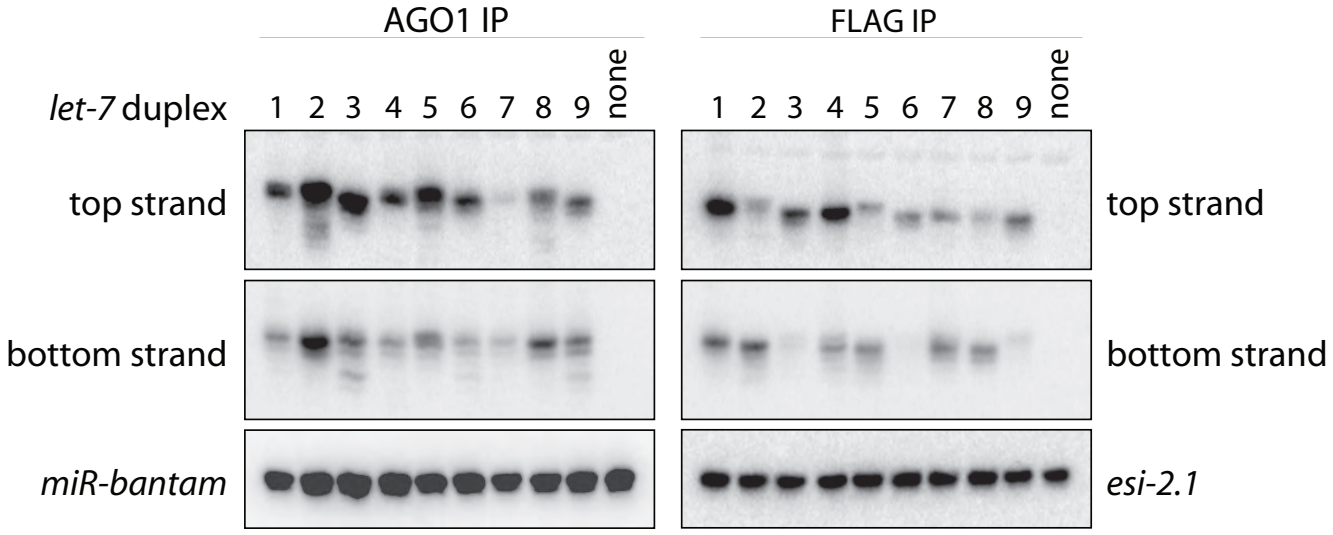

AGO1 IP
FLAG IP
let-7 duplex
1 2 3 4 5 6 7 8 9 none
1 2 3 4 5 6 7 8 9 none
top strand
bottom strand
miR-bantam
top strand
bottom strand
esi-2.1

Figure S4. The impact of *dcr-2* knockdown on miR* loading into AGO2.

The fraction of miR strands within all AGO1-associated miRNAs was analyzed from small RNA libraries of untreated cells or cells depleted of Dcr-2 (grey). In parallel, the percentage of AGO2-bound miR* species within all miRNA reads was plotted for the same conditions (black).

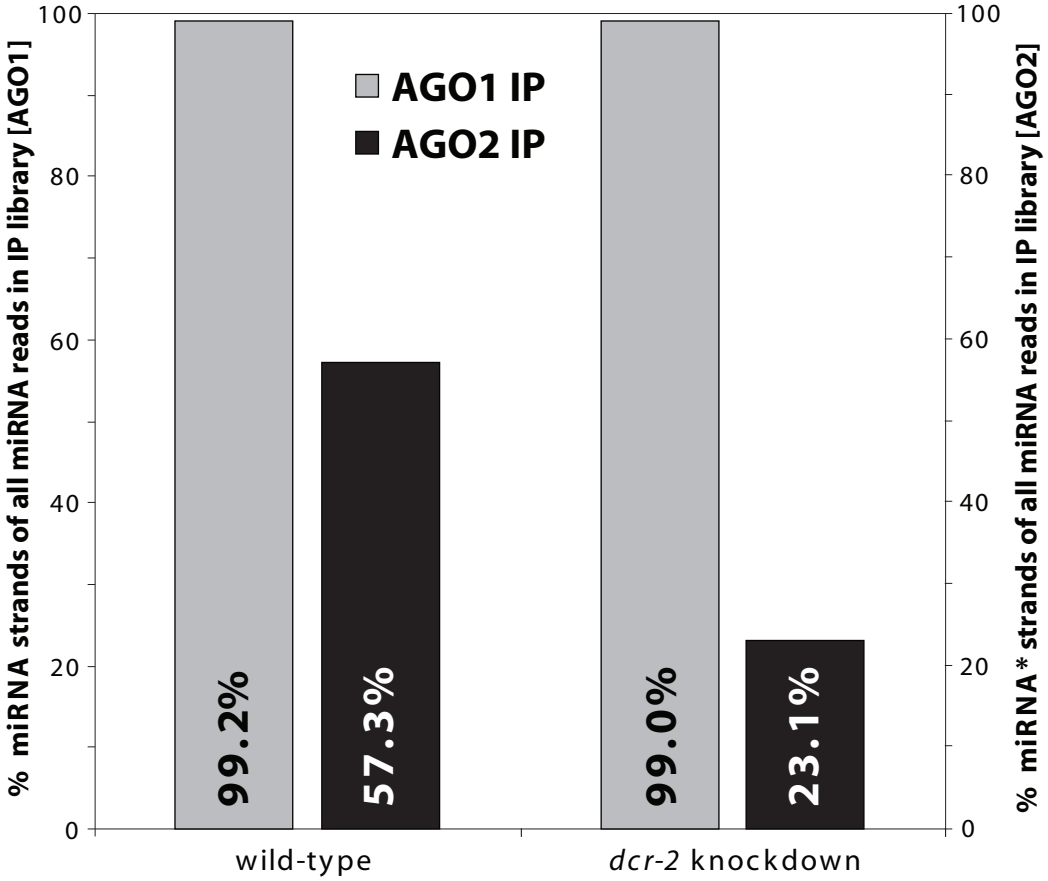

AGO1 IP
AGO2 IP
% miRNA strands of all miRNA reads in IP library [AGO1]
% miRNA* strands of all miRNA reads in IP library [AGO2]
100
80
60
40
20
0
99.2%
57.3%
99.0%
23.1%
wild-type
dcr-2 knockdown

Figure S5. Effects of Dcr-1 or AGO1 depletion on *miR-276a* and *miR-276a sensors in flies.**

Shown are sensors for *miR-276a* or *miR-276a** featuring perfectly matched or bulged target sites (as indicated to the left). **(A-D)**. Negative β-Gal staining (red in the merged images) indicates *dcr-1* mutant clones (also marked with arrows). Cells with strong β-Gal staining contain two wild-type *dcr-1* genes, while cells with intermediate staining are heterozygous for *dcr-1*. EGFP sensor activity is shown in green. The black and white panels indicate the separate channels for β-Gal and EGFP. (**E-H**) Clonal analysis for *ago1*: Details as in **(A-D)**. Selected regions (enclosed in white boxes) were zoomed in and shown as insets within each panel to visualize the smaller *ago1* clones.

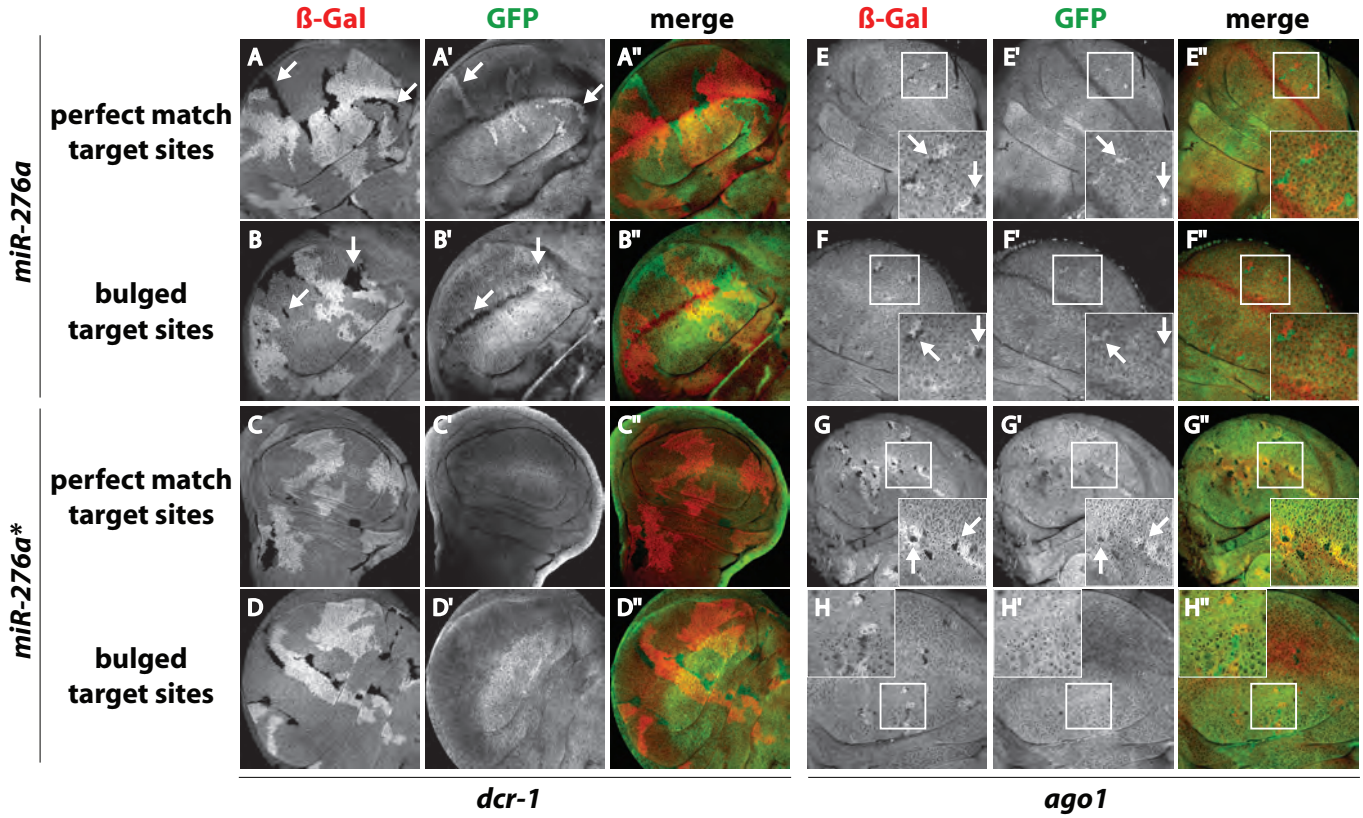

ß-Gal
GFP
merge
ß-Gal
GFP
merge
miR-276a
perfect match target sites
bulged target sites
miR-276a*
perfect match target sites
bulged target sites
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
H
H'
H"
dcr-1
ago1

Figure S6. Effects of AGO2 depletion on microRNA and microRNA* sensors in flies.

Shown are sensors for the miRNA strand or miRNA* strand of *miR-276a* **(A-D)** or *miR-bantam* (**E-H**) featuring perfectly matched or bulged target sites (as indicated to the left) in *ago2* clones.

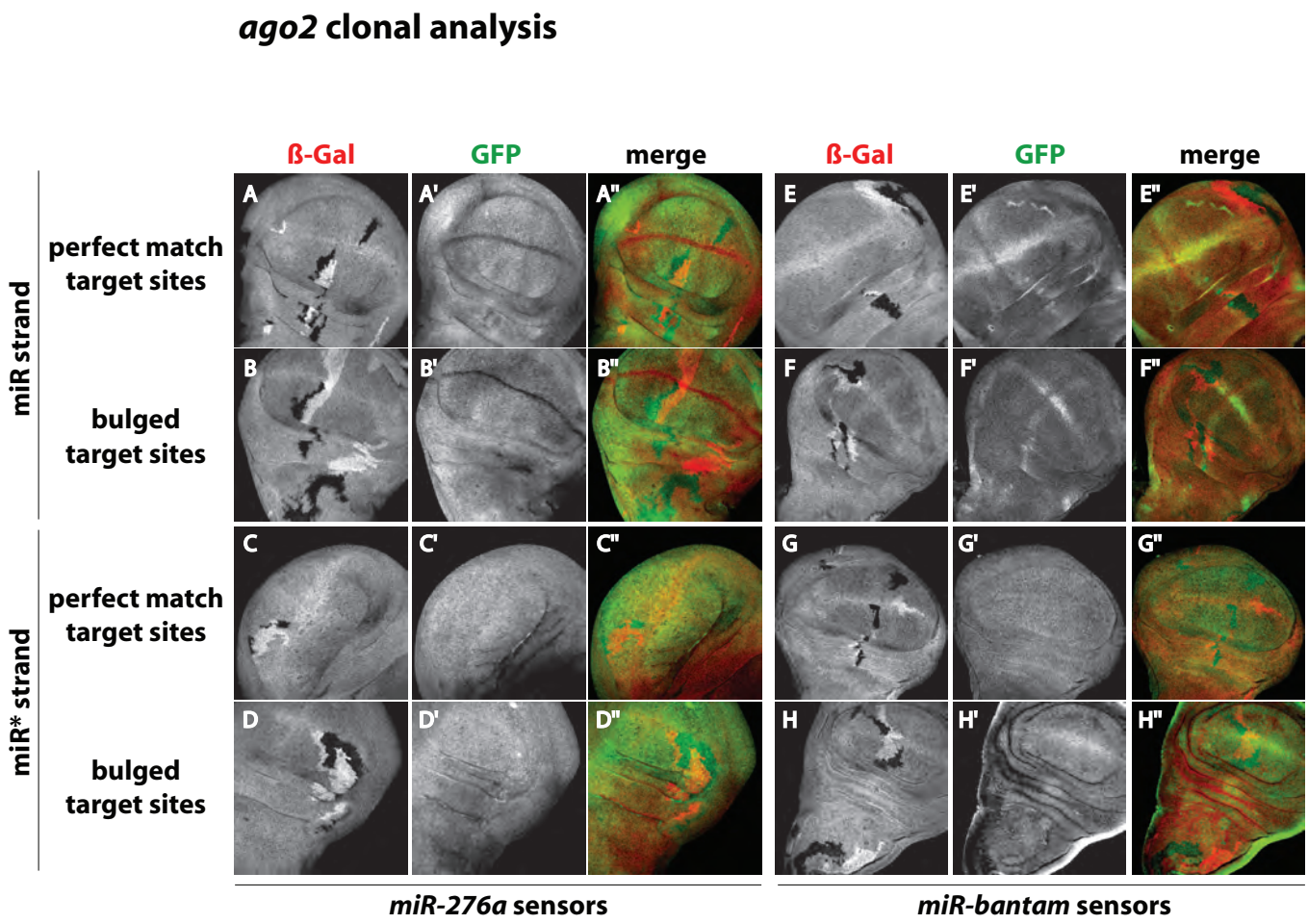

ago2 clonal analysis
ß-Gal
GFP
merge
ß-Gal
GFP
merge
miR strand
perfect match target sites
bulged target sites
miR* strand
perfect match target sites
bulged target sites
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
H
H'
H"
miR-276a sensors
miR-bantam sensors

Figure S7. Effects of Dcr-2 depletion on microRNA and microRNA* sensors in flies.

Shown are sensors for the miRNA strand or miRNA* strand of *miR-276a* **(A-D)** or *miR-bantam* (**E-H**) featuring perfectly matched or bulged target sites (as indicated to the left) in *dcr-2* clones.

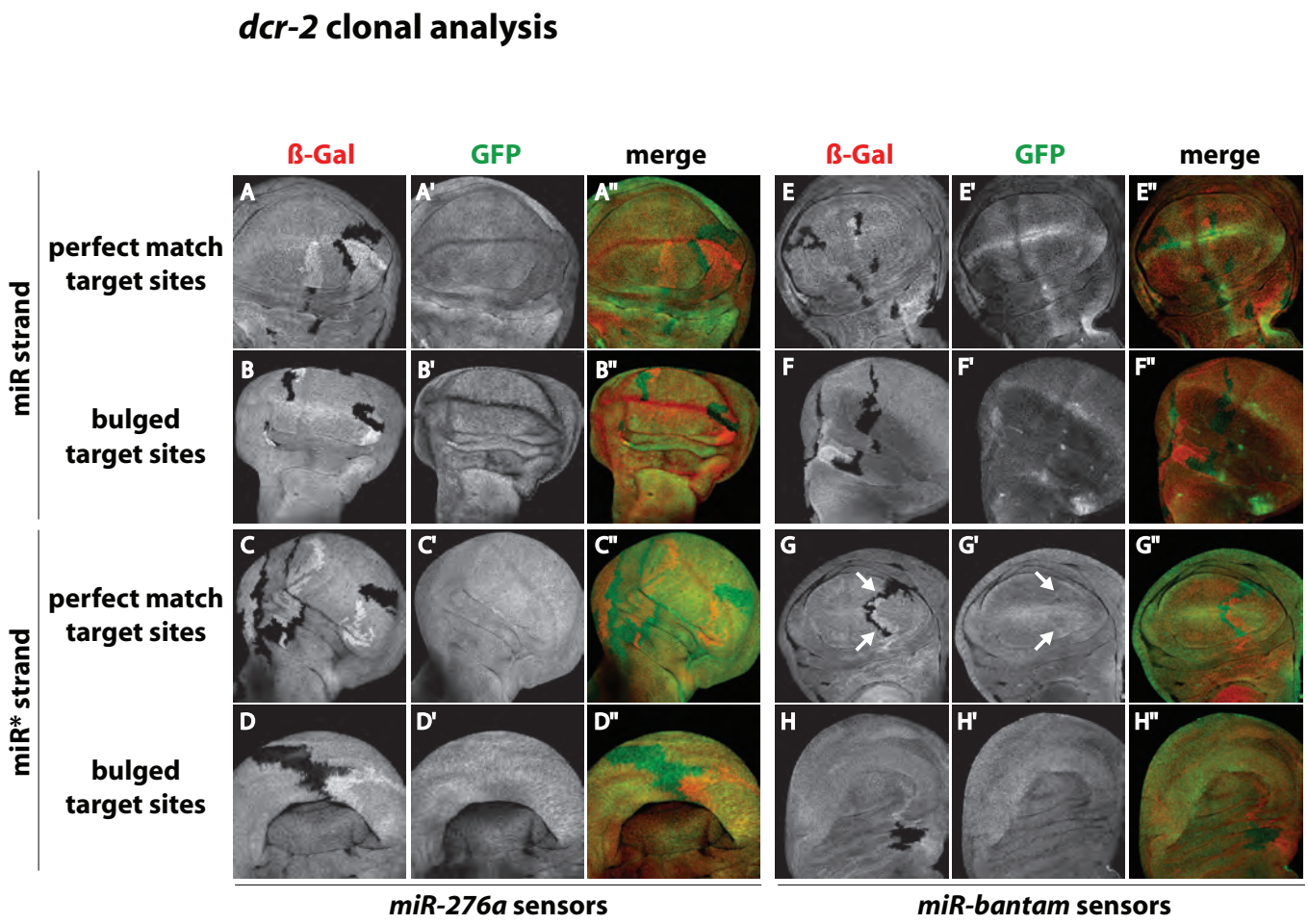

dcr-2 clonal analysis
ß-Gal
GFP
merge
ß-Gal
GFP
merge
miR strand
perfect match target sites
bulged target sites
miR* strand
perfect match target sites
bulged target sites
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''
E
E'
E''
F
F'
F''
G
G'
G''
H
H'
H''
miR-276a sensors
miR-bantam sensors

Figure S8. Effects of R2D2 depletion on microRNA and microRNA* sensors in flies.

Shown are sensors for the miRNA strand or miRNA* strand of *miR-276a* **(A-D)** or *miR-bantam* (**E-H**) featuring perfectly matched or bulged target sites (as indicated to the left) in *r2d2* clones.

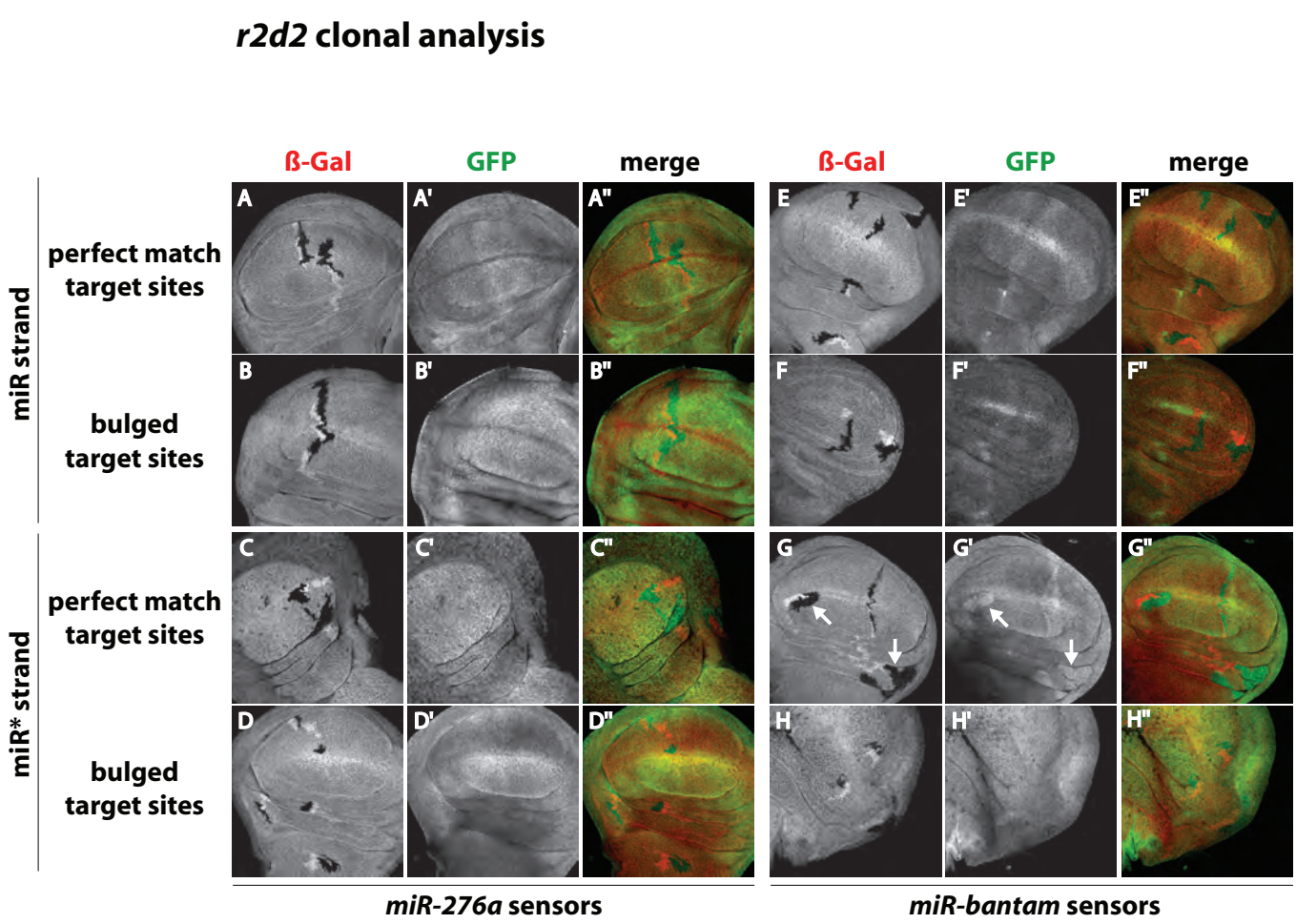

r2d2 clonal analysis
ß-Gal
GFP
merge
ß-Gal
GFP
merge
miR strand
perfect match target sites
bulged target sites
miR* strand
perfect match target sites
bulged target sites
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
H
H'
H"
miR-276a sensors
miR-bantam sensors

Figure S9. Effects of Loqs depletion on on microRNA and microRNA* sensors in flies.

Shown are sensors for the miRNA strand or miRNA* strand of *miR-276a* **(A-D)** or *miR-bantam* (**E-H**) featuring perfectly matched or bulged target sites (as indicated to the left) in *loqs* clones.

loqs clonal analysis

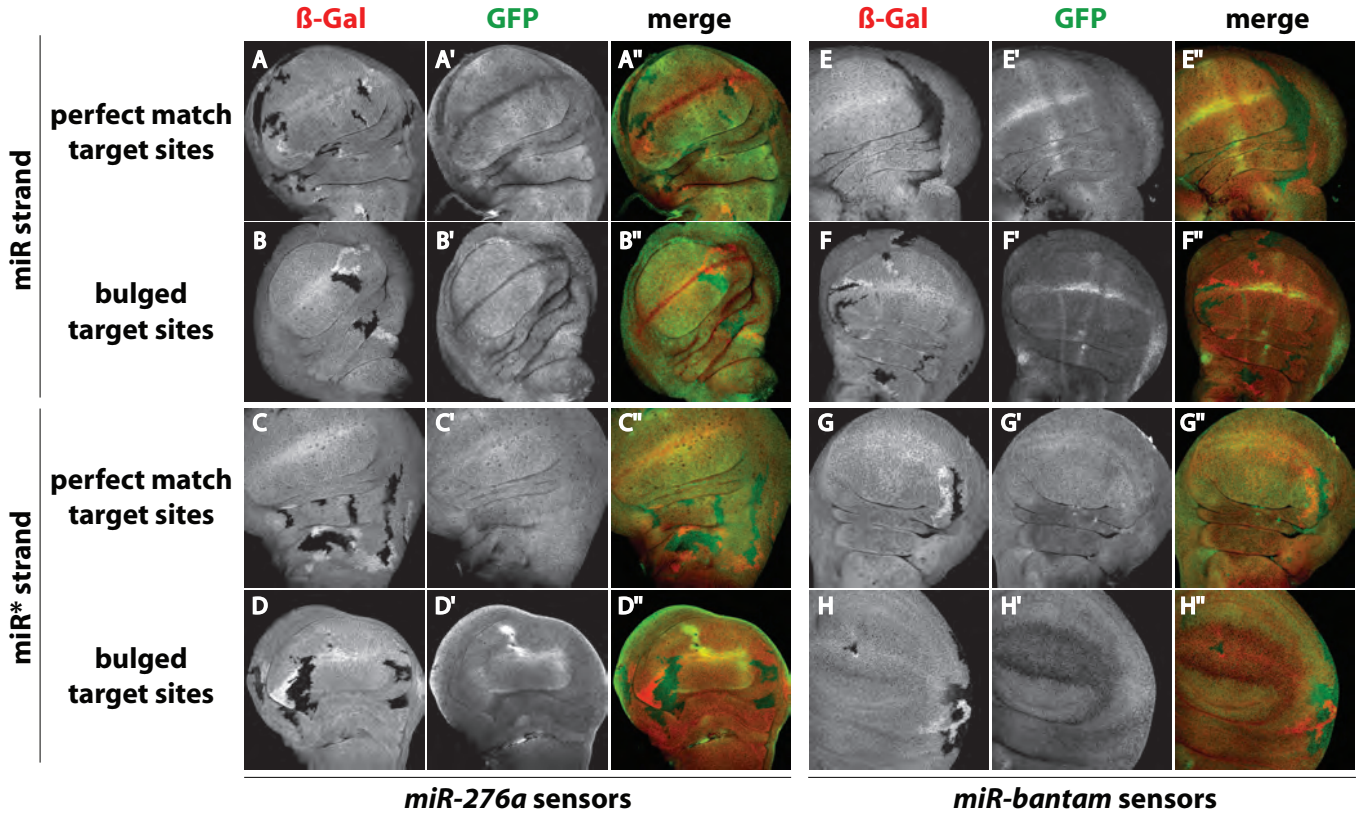
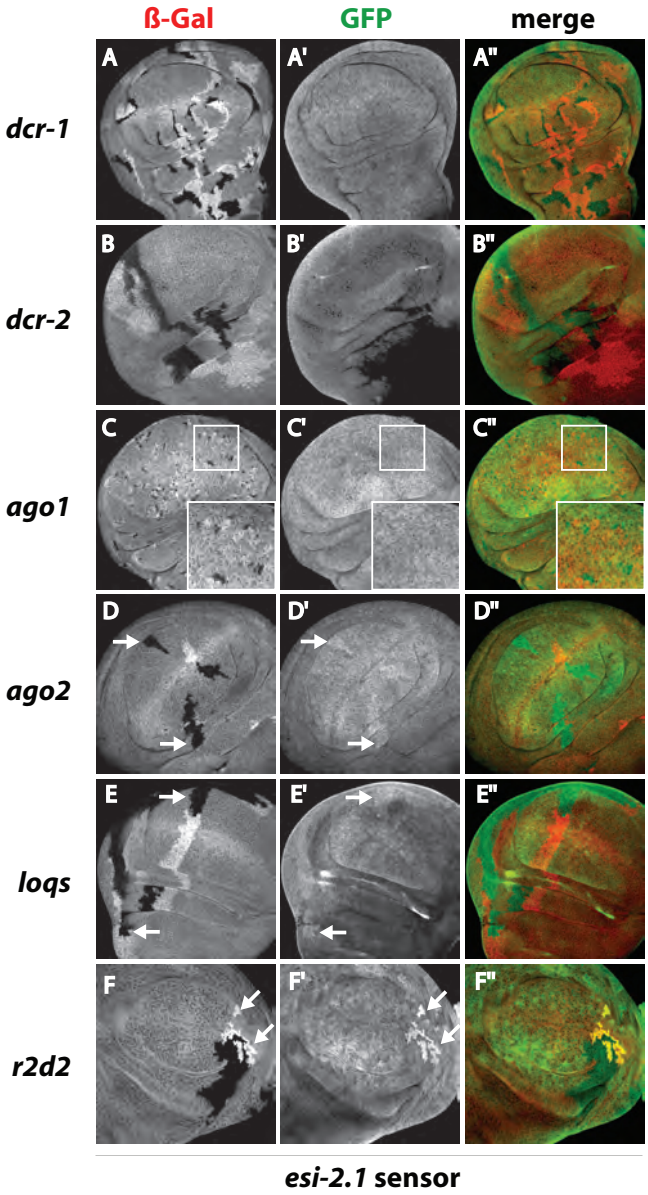


Figure S10. Effects of depletion of canonical miRNA and siRNA pathway components on *esi-2.1* sensor in flies.

Shown are sensors for *esi-2.1* featuring perfectly matched target sites in mitotic clones carrying mutations in genes encoding canonical miRNA and siRNA pathway components. **(A)** *dcr-1*; **(B)** *dcr-2*; **(C)** *ago1*; **(D)** *ago2*; **(E)** *loqs*; **(F)** *r2d2*.


ß-Gal
GFP
merge
dcr-1
dcr-2
ago1
ago2
loqs
r2d2
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
esi-2.1 sensor